THE EFFECT OF INHIBITING KPNB1-MEDIATED NUCLEAR IMPORT ON CANCER CELL BIOLOGY AND INFLAMMATORY TRANSCRIPTION FACTOR SIGNALLING

Tamara Stelma

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DECLARATION

I, Tamara Stelma, hereby declare that the contents of this thesis is my own unaided work, except where acknowledgements indicate otherwise; and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I am now presenting this thesis for academic examination towards the Degree of Doctor of Philosophy in Medical Biochemistry.

Signed by candidate

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ABBREVIATIONS

%	Percentage
°C	degrees Celsius
Ab	Antibody
AMPS	2-Acrylamido-2-methylpropane sulfonic acid
AP-1	Activating protein 1
APC	Adenomatous Polyposis Coli
ATCC	American Tissue Culture Collection
ATF	Activating Transcription Factor
BAF	B-cell Activation Factor
BCR	B-Cell Receptor
BCR-ABL	Breakpoint Cluster Region - Abelson
bps	Base pairs
BSA	Bovine Serum Albumin
BSL2	Bio-Safety Level 2
C	Control
CBA	Cytometric Bead Array
CDK	Cyclin Dependent Kinase
cDNA	Complimentary deoxyribonucleic acid
CI	Confidence Interval
cm	Centimetre
Cox-2	Cyclooxygenase 2
CSE1L	Chromosome Segregation 1 Like protein
CTLA-1	Cytotoxic T-Lymphocyte-associated Antigen 1
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
dH_2O	Deionised water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dsRNA	Double-stranded ribonucleic acid
DTT	Dithiothreitol

ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Egtazic acid
EMT	Epithelial-to-Mesenchymal Transition
ERK	Extracellular signal-Regulated Kinase
FCS	Foetal Calf Serum
FDA	Food and Drug Administration
FRA1	Fos-related Antigen 1
G	Gravitational force
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte Macrophage- Colony Stimulating Factor
H&E	Haematoxylin and Eosin
H_2O	Water
HEN-1	Small RNA 2'-O-methyltransferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HER2	Human Epidermal Growth factor Receptor 2
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
hr/s	Hour/s
HURP	Hepatoma up-regulated protein
i.p.	Intraperotoneal
ICAM-1	Intracellular Adhesion Molecule 1
IFN-α	Interferon alpha
IFN-γ	Interferon gamma
IgG	Immunoglobulin G
IKK	IκB Kinase
IL	Interleukin
INI-43	Inhibitor of Nuclear Import 43
IκB	Inhibitor of kappa B
JNK	c-JUN N-terminal Kinase
KD	Knockdown
KDa	Kilodalton

kg	Kilogram
KPNA	Karyopherin alpha
KPNB1	Karyopherin beta 1
Log	Logarithm
LPS	Lipopolysaccharide
LTβR	Lymphotoxin β-receptor
M	Molar
MAF	Musculoaponeurotic Fibrosarcoma
MAPK	Mitogen Activated Protein Kinase
mg	Milligram
mins	Minutes
ml	Millilitre
mm	Millimetre
mM	Millimolar
MMP	Matrix Metalloprotease
MOPS	3-morpholinopropane-1-sulfonic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NBS1	Nibrin
NES	Nuclear Export Signal
NFAT	Nuclear Factor of Activated T-cells
NFkB	Nuclear Factor kappa B
NFY	Nuclear transcription Factor Y
ng	Nanogram
NIK	NFkB Inducing Kinase
NLS	Nuclear Localisation Signal
nm	Nanometre
nM	Nanomolar
NPC	Nuclear Pore Complex
NRF-2	Nuclear factor (erythroid-derived 2)-like 2 protein
NuMA	Nuclear Mitotic Apparatus protein
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed Death 1

PI	Protease inhibitors
PMA	Phorbol-12-Myristate-13-Acetate
PSA	Prostate Specific Antigen
PTHrP	Parathyroid Hormone-related Protein
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RanGDP	Ran Guanosine Diphosphate
RanGTP	Ran Guanosine Triphosphate
Rb	Retinoblastoma protein
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
rpm	Revolutions per minute
RREB-1	Ras Responsive Element Binding Protein 1
RT	Room Temperature
SD	Standard Deviation
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SINE	Selective Inhibitors of Nuclear Export
siRNA	Small interfering RNA
Sp1	Specificity Protein 1
SREPB2	Sterol Regulatory-related Protein B2
STAT	Signal Transducer and Activator of Transcription
TAMs	Tumour Associated Macrophages
TBE	Tris Borate EDTA
TBP	TATA-Binding Protein
TBS	Tris Buffered Saline
TCR	T-Cell Receptor
TEMED	Tetramethylethylenediamine
TIMP	Tissue Inhibitor of Matrix metalloproteases
TNF-α	Tumour Necrosis Factor alpha
TPX2	Microtubule Nucleation Factor
U/ml	Units per millilitre
UCT	University of Cape Town
uPA	Urokinase-type Plasminogen Activator

uPAR	Urokinase-type Plasminogen Activator Receptor
UV	Ultra Violet
V	Volts
VCAM-1	Vascular Cell Adhesion Molecule 1
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation
Wt1	Whilms' Tumour protein
XPO1	Exportin 1
µg	Microgram
µl	Microlitre
µm	Micrometre
µM	Micromolar

ABSTRACT

Cancer remains one of the major causes of morbidity and mortality globally. Many novel and innovative approaches have been employed to develop new chemotherapeutic strategies, of which targeted therapies aim to identify a molecular lesion or dysregulated pathway that cancer cells are dependent on. Research in our laboratory and others identified the nuclear import protein, Karyopherin β1 (KPNB1), to be overexpressed in various cancers and that inhibiting its expression blocks the proliferation of cancer cells. However, little is known about the potential role of KPNB1 in other cancer cell phenotypes and inflammatory signalling pathways. The aim of this study was to investigate the anticancer and anti-inflammatory effects of inhibiting nuclear import via KPNB1 and to characterise the *in vivo* effect of the small molecule inhibitor of nuclear import, INI-43, on tumour formation. Using siRNA and a small molecule inhibitor, INI-43, to inhibit KPNB1 we found that cervical cancer cell migration and invasion was significantly reduced. The reduced motility of cancer cells was associated with a decrease in MMP-2 and -9 expression and an increase in TIMP-1 and -2 expression following INI-43 treatment. This corresponded with a decrease in MMP-9 gelatinase activity in KPNB1-inhibited cervical cancer cells. Extended periods of KPNB1 inhibition lead to decreased proliferation and apoptosis.

These changes in cancer cell biology when KPNB1 is inhibited may in part be due to its function as a nuclear transporter of transcription factors associated with cancer cell proliferation, migration and invasion. We therefore investigated the effects of KPNB1 inhibition on the nuclear localisation and transcriptional activity of key transcription factors; NFkB and AP-1, both having been implicated in many of the hallmarks of cancer. Immunofluorescent analysis

and nuclear/cytoplasmic fractionation assays showed that KPNB1 inhibition blocked the nuclear localisation of NFkB. Electromobility shift assays confirmed a reduced NFkB binding to an NFkB DNA-binding sequence in the nuclear extract of KPNB1-inhibited cells. Luciferase reporter assays containing NFkB and/or AP-1 consensus binding sites showed reduced transcriptional activity for both transcription factors following KPNB1 inhibition. Associated with these changes in NFkB and AP-1 activity was reduced inflammatory cytokines; IL-6, IL-1β, TNF-α and GM-CSF target gene expression.

To further characterise the role of INI-43 as a potential chemotherapeutic, the effects on tumour growth and development were investigated in an ectopic xenograft mouse model. INI-43 treatment significantly reduced tumour growth in mice and associated with the redistribution and reduction in KPNB1 levels. INI-43 treated tumours also showed altered morphological features including; better tissue differentiation and reduced inflammatory stromal infiltration, as well as reduced Ki-67 expression. The expression of extracellular matrix components and the cytoskeletal structure of cancer cells was analysed to further investigate the role of KPNB1 inhibition in tumour development. Inhibition of KPNB1 in cancer cells caused reduced expression of both collagen type IV and MMP-9. The redistribution of B-catenin and F-actin suggested that INI-43 treatment caused a loss of mesenchymal features required for tumour progression.

The nuclear transport system has been of particular interest in recent years for the development of targeted anticancer drugs. However, most studies have focused on nuclear export inhibitors with little known on the potential of nuclear import inhibitors as anticancer drugs. This study provides evidence that inhibiting the nuclear import protein, KPNB1, has anti-inflammatory and anticancer effects and shows promise as an anticancer approach requiring further investigation.

CHAPTER 1

LITERATURE REVIEW

1.1 Cancer burden

Cancer remains one of the major causes of morbidity and mortality globally. The latest World Cancer Report of 2014 together with GLOBOCAN 2012 detail the global impact of cancer [1, 2]. In 2012, 14.1 million new cancer cases were diagnosed with 8 million cancer-related deaths. It is predicted that this statistic will rise to over 20 million new annual cancer cases by 2025. Cancer is a multi-factorial disease with an ageing and growing population contributing to the increased incidence and demographic distribution of cancer cases despite advances in prevention, diagnosis and therapy for cancer. Among the most common cancers globally including; lung, breast, prostate and colorectal and cervical cancer which is the fourth most common cancer among woman. Statistics for low to middle income countries however, show that cervical cancer is still often the most common cancer among woman. Risk factors for cancer include what are considered as “lifestyle choices” such as tobacco use, obesity, infections and sun exposure as well as non-modifiable risk factors including; familial genetic background, race or reproductive and hormonal history. Carcinogen exposure also includes; air/water pollution, foods, drugs or other consumer products. This adds to the challenge of developing cancer prevention strategies. Infection is considered a strong risk factor for specific cancers including cervical cancer. In Sub-Saharan Africa, 33% of cancer cases in 2008 were attributable to infections which is the highest globally. In countries like South Africa infection with HIV can substantially increase the risk of virus-associated cancers due to immunosuppression. One such cancer is cervical cancer. HIV infection is known to increase

the risk of HPV-related neoplasias [3-6]. These statistics highlight the necessity for continued research in the field of oncology. Understanding cancer cell biology is essential for the development of rationally designed, targeted therapy and other preventative and therapeutic strategies.

1.2 Conventional therapeutic strategies for cancer and their limitations

Early treatment is the determining factor for patient survival. The success of such treatment may vary greatly depending on the type and stage of cancer, the patient's age, health condition and medical history. The ultimate goal of treatment is cure but it also has advantages in palliation, prolonging survival and preventing metastasis. The current approach for the management of solid tumours includes i) surgery, ii) chemotherapy and iii) radiation therapy.

i) Surgery

This form of cancer therapy is generally the first line of approach should the nature of the tumour allow for it. It involves surgically removing solid tumours and surrounding tissue to prevent possible dissemination. Surgery is normally performed in conjunction with chemotherapy and/or radiation therapy. This technique is limited to self-contained, solid tumours as it has little use for haematological cancers or disseminated tumours.

ii) Chemotherapy

Conventional cancer chemotherapeutics are targeted towards actively dividing cells. These drugs usually disrupt the cell cycle either in a phase-specific or non-specific manner but can also utilise other mechanisms of action. The first type of chemotherapeutic drugs belonged to the class of alkylating agents and begun use in the early 1940's. Alkylating agents act at any

stage in the cell cycle and cause irreparable DNA damage which leads to cell death. Several categories of compounds fall into this class including the metal salts such as Cisplatin [7]. Two other common classes of chemotherapeutics are; anti-metabolites and natural products. Anti-metabolites are folic acid, pyrimidine and purine analogues; common components used in nucleic acid synthesis. These drugs normally cause cell death during S phase of the cell cycle when they are incorporated into RNA and DNA or inhibit enzyme function required for nucleic acid production. Methotrexate is a common example of an anti-folate while 5'-fluorouracil is a pyrimidine analogue [8]. Natural products include a range of taxanes, topoisomerase inhibitors and anthracyclines (antibiotics). The antibiotic, Doxorubicin, is among the most effective and commonly used chemotherapeutic agents against solid tumours [9]. While the goal of chemotherapy is to cure the cancer, prolong survival, provide palliation and radiosensitise cancer cells it is often at the cost of severe toxic side-effects. Many of the conventional chemotherapeutics are hydrophobic, requiring the use of solvents, which increases their toxicity [10]. Their cytotoxicity is also not selective for cancer cells and severe side-effects are not uncommon. These limitations including the development of drug resistance emphasises the need for more effective and specific chemotherapeutic strategies.

iii) Radiation therapy

This technique uses high-energy radiation such as X-rays, gamma rays and charged particles to shrink tumours and kill cancer cells by causing DNA damage [11]. Radiation therapy is often used in combination with surgery and/or chemotherapy. As with surgery this technique is also limited to solid tumours and is not particularly useful for haematological cancers and disseminated tumours. Although radiation therapy can be isolated to a specific region of tissue it can still cause damage to normal cells.

1.3 Developing new chemotherapeutic strategies

Many novel and innovative approaches have been employed to develop new chemotherapeutic strategies against cancer cells. These approaches may not all seek to replace current strategies but also to incorporate them into combination therapies either with each other or with conventional chemotherapeutic approaches. The following areas of research; personalised medicine, targeted therapies and immunotherapy are making significant contributions to the field of chemotherapeutics in recent years.

i) Personalised medicine

Personalised medicine is also referred to as precision medicine or genomic medicine. This relatively new therapeutic strategy is generating a wealth of information with potential for cancer prevention and treatment. The first full genome sequence was obtained in 2003. Through sequencing the genome, it has been proposed that treatment decisions can be made on the tumours genetic signature rather than based on tumour tissue type or anatomical origin. The genetic makeup of individuals is unique and similarly for their tumours therefore it is predicted that personalising treatment may maximise efficiency while minimising toxicity [12]. Genetic alterations in genes associated with cancer such as; HER2 in breast cancer, KRAS in colorectal cancer and BCR-ABL in chronic myeloid leukaemia, highlight the value of genetic screening in treatment decision making [13-15]. The Personalized Oncogenomics Program which is run by The BC Cancer Agency started in 2012 and incorporates genomic sequencing into real-time treatment planning for a cohort of patients with late stage incurable cancers. This oncogenomics trial has to date enrolled 750 patients and has shown promise for this approach in future chemotherapeutic strategies [16]. The high cost associated with such an approach as well as the timeous turnaround time are still limiting factors which need to be improved.

ii) Small molecules in targeted cancer therapy

The Human Genome Project which enabled the sequencing of DNA has led to the advance of technology that can detect genomic, transcriptomic, proteomic and epigenetic alterations. This has aided in the development of targeted therapies with potential for great success. The basic approach of targeted therapy is to identify a molecular lesion or pathway that cancer cells are dependent on and develop an appropriate drug to inhibit its function. This process often takes advantage of the oncogenic addiction pathways in cancer. Targeted therapy received prominence in the late 1990's with the introduction of the small molecule inhibitors; Imatinib targeting BCR-ABL in chronic myeloid leukaemia and Getfitinib targeting the EGF receptor in non-small cell lung cancer [17-19]. A variety of small molecule inhibitors have been approved by the FDA to date and include targets such as CD20 for non-Hodgkin's lymphoma, EGFR for colorectal cancer, CD33 and CD52 for leukaemia and VEGF for colorectal and non-small cell lung cancer [20]. The advantage with small molecules is their ability to target proteins located within the cellular compartment in addition to proteins on the surface of cancer cells.

Transcription factors found to be overactive in cancer may also make potential targets for anticancer drugs. They were previously thought of as "undruggable" targets because of the relatively large interacting surface between the transcription factor and the DNA [21]. These proteins are activated through signal transduction pathways beginning with activation of a receptor by a ligand, a cascade of intracellular signalling leading to the translocation of the transcription factor into the nucleus where it can bind the DNA and initiate gene expression. Points of interception in these signalling pathways may include receptor-ligand binding, kinase activity, nuclear transport machinery or DNA-binding ability (Fig. 1.1).

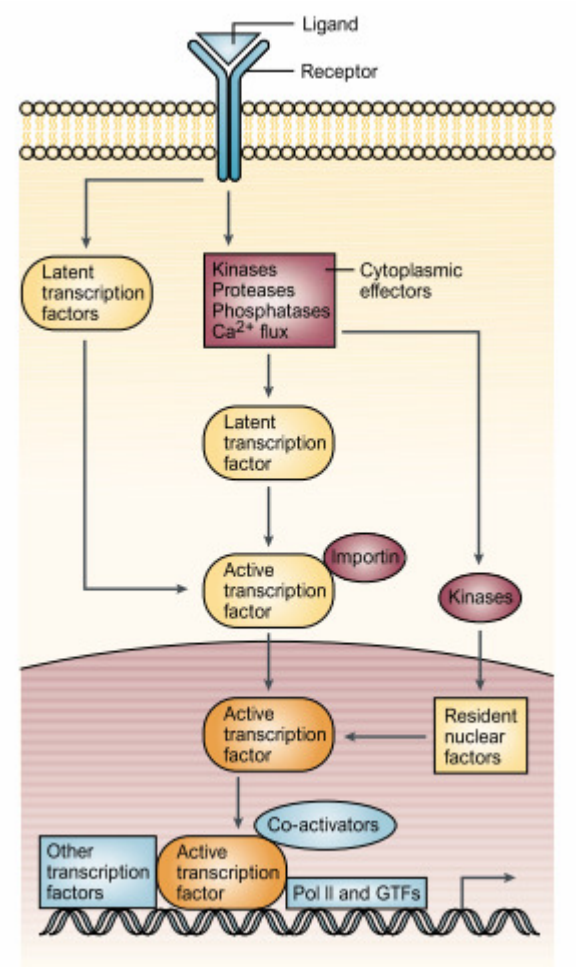


Figure 1.1: A generalised signal transduction pathway. Key reactions are summarised in the diagram that form possible points of interception when designing targeted therapies From Darnell, 2002.

The major families considered as targetable transcription factors in cancer include 1) NFkB and AP-1 families, 2) STAT family and 3) Steroid receptors [22]. Further detail on NFkB and AP-1 transcription factors and their contribution to cancer will be discussed in sections 1.6 and 1.7. A feature essential for transcription factors is their translocation from the cytosol into the nucleus where they have function. Darnell (2002) proposed that inhibiting nuclear transport may be a reasonable target to inhibit several transcription factors that are overactive in cancer cells [23].

While targeting a single dysregulated protein has value in many cancers it may be less useful in cancers originating from carcinogen-induced genetic aberrations where there are multiple competing molecular signalling pathways. (e.g. tobacco-induced lung cancer or UV-induced melanoma). This emphasizes that in some cases more than one pathway needs to be targeted or a combination treatment strategy needs to be employed.

iii) Immunotherapy

The role of the immune system in preventing and controlling cancer has long been studied but only in the last two decades have there been new therapeutic strategies developed to use the immune system to target cancer tumours. Immunotherapy involves one of two strategies either targeting the tumour cells directly or activating the immune cells to in turn target the cancer cells. Targeting the tumour directly has been successfully achieved through the use of monoclonal antibodies. These monoclonal antibodies are designed to bind specific receptors or antigens on the surface of cancer cells, marking them for immune destruction. In 1997 the first monoclonal antibody, Rituximab, was used to treat lymphoma [24]. Since then many monoclonal antibodies have been approved by the FDA for the treatment of cancer. An example includes; Trastuzumab targeting the HER2 receptor in breast cancer [20]. This technology has the potential to conjugate radioisotopes or other cytotoxic molecules to the antibodies to achieve targeted delivery to the tumour and enhanced effectiveness [25-27]. New surface receptors and cancer-specific antigens are continually being discovered presenting opportunities for the design of new monoclonal antibodies as therapeutics.

Therapies designed to activate the immune system include cancer vaccines, check point inhibitors and immune system boosters. Cancer vaccines aim to induce anti-tumour T-cells by immunising patients with either tumour-specific or tumour-associated antigens. To date only one cancer vaccine has been approved by the FDA. Sipuleucel-T used for the treatment of a

type of metastatic prostate cancer [28]. Nonetheless successful clinical trials using cancer vaccines still provides hope for their use as a therapeutic strategy. A second strategy is to simply enhance the function of pre-existing anti-tumour T cells. The tumour develops several strategies to evade the host immune response and these are collectively known as immune check points. These immune check points can render endogenous anti-tumour T-cells ineffective [29]. Specific checkpoint inhibitors could override this immune evasion restoring function to these T-cells. Two successful checkpoint targets have been cytotoxic T-lymphocyte-associated antigen 1 (CTLA-1) in patients with metastatic melanoma and the programmed death 1 (PD-1) pathway in patients with relapsed or refractory Hodgkin's Lymphoma [30, 31]. Lastly immune system boosters can be used in the form of cytokines or immunomodulating drugs. Cytokines are naturally produced by cells of the immune system and modulate immune and inflammatory reactions within the body. The use of interferon alpha (IFN-α) and interleukin 2 (IL-2) have both been successful in the treatment of a small subset of melanoma patients [32]. The upregulation of a widespread T-cell response can unfortunately lead to non-specific side effects.

1.4 Hallmarks of cancer

The development of cancer is associated with an accumulation of genetic alterations leading to the gain of function of genes promoting cancer (oncogenes) and the loss of function of genes (tumour suppressor genes) preventing it. Hanahan and Weinberg (2000) initially described six essential alterations in cell physiology that together are responsible for malignant growth [33]. In a subsequent review, two additional hallmarks of cancer were added as well as two enabling factors critical for the development of cancer (Fig. 1.2) [34]. The eight hallmarks of cancer are:

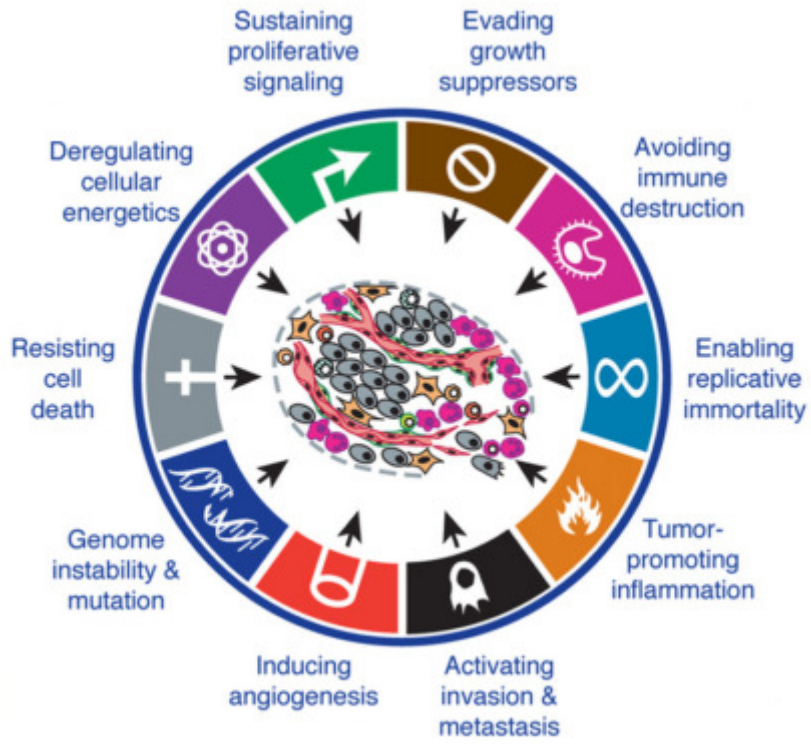


Figure 1.2: The Hallmarks of cancer including the acquired characteristics and enabling factors. From Hanahan and Weinberg, 2011.

i) Sustaining proliferative signalling

Possibly one of the most essential traits of cancer cells is the ability to sustain proliferation. In normal cells, growth signals are tightly controlled by mitogenic factors that instruct cells to move from a quiescent state to a proliferative one. Cancer cells lose their dependence on exogenous growth signals and are thought to produce their own which allows them to be in control of their own proliferative state. This process is mediated by various oncogenes.

ii) Evading growth suppressors

Antigrowth signals normally exist to counter balance the growth promoting signals. They can act by either forcing actively proliferating cells into a quiescent state or by permanently

relinquishing their proliferative potential. Cancer cells have to overcome these anti-proliferative signals in order to succeed. Disruption of the retinoblastoma protein (pRb) or the tumour suppressor protein, p53, are most commonly responsible for the ability to evade anti-growth signals.

iii) Resisting cell death

The carefully orchestrated cascade of events leading to cell death by apoptosis is essential for removing damaged cells. The alteration of tumour suppressor genes in cancer cells allows them to evade cell death.

iv) Enabling replicative immortality

The combined effects of self-sufficiency from growth signals, evading growth suppressors and resisting cell death lead to an unlimited ability of cancer cells to replicate. This allows tumours to continue expanding beyond the confines of their environment.

v) Activating invasion and metastasis

Cancer cells acquire invasive properties in order to escape the confines of the primary site and occupy secondary locations. This is accomplished by epithelial-to-mesenchymal transition (EMT), a complex network of events that occurs in cancer cells at the invasive front of the tumour. The cells in this position receive stimuli from the surrounding microenvironment that drive these phenotypic changes. Epithelial cancer cells begin to lose their adherens junctions and expression of epithelial genes such as E-cadherin. While increasing the expression of mesenchymal genes such as vimentin and taking on a more spindle-shaped morphology. This is accompanied by the increased expression of matrix-degrading enzymes, enhancing motility, as well as a heightened resistance to apoptosis. Once cancer cells obtain these abilities they

are able to intravasate and disseminate to a secondary location leading to metastasis. Metastasis associates with a poor prognosis in many cancers.

vi) Inducing angiogenesis

As tumours grow their demand for nutrition and oxygen exceeds the supply and the growth of new blood vessels needs to be stimulated in order to compensate for this. Cancer cells are capable of producing signals that induce angiogenesis.

vii) Avoiding immune destruction

Essentially cancer cells are not foreign to the human body. Although the immune system is on high alert for developing cancer cells they can produce various signals modulating the immune response and thereby escape detection and destruction.

viii) Deregulating cellular energetics

The development of chronic proliferation needs to be accompanied by an adjustment in energy metabolism to sustain the proliferative demands of these cells. Cancer cells favour glycolysis to be able to still produce for their biomass creation and meeting their energy needs under limited oxygen conditions.

In addition to these hallmarks of cancer two enabling characteristics have been described which contribute to the acquisition of these hallmarks. These are tumour-promoting inflammation and genomic instability. The tumour microenvironment is infiltrated with host immune cells and while previously this was thought to play a role in tumour eradication in fact the opposite was true and a state of inflammation was found to fuel cancer initiation and progression. The development of genomic instability arises from an accumulation of random

mutations that confer an advantage in cancer cells by assisting in acquiring the cancer hallmarks.

1.5 Inflammation and cancer

Inflammation forms part of the innate immune response and acts typically in a normal host response to bacterial or viral infection as well as being critical in the process of wound healing. Tissue injury triggers a cascade of events involving the activation and recruitment of immune cells such as monocytes, neutrophils and eosinophils to the site of injury. Neutrophils are normally the first immune cells to enter the site of injury from the surrounding vasculature while macrophages, formed from monocytes in tissue, follow closely after. Macrophages are the main source of chemokines, cytokines and growth factors which severely influence the neighbouring tissue. Chemokines are responsible for the recruitment of specific immune effector cells and components, which dictate the progression of the inflammatory process. Cytokines can be pro-inflammatory or anti-inflammatory and maintain a critical balance in wound healing. Although wound healing and inflammation are normally self-limiting processes a disruption in the balance of pro-inflammatory and anti-inflammatory signals could result in a chronic inflammatory state [35].

Inflammation was first linked to cancer in the 19th century when Virchow discovered leucocytes present in tumours [36]. In more recent years there has been extensive research investigating the connection between inflammation and cancer. A chronic inflammatory environment where there is sustained proliferation of cells, high concentrations of inflammatory cells, cytokines, chemokines, growth factors and DNA-damage promoting agents can potentially promote neoplastic formation [37]. Wound healing can fail to terminate when the highly susceptible proliferating cells sustain DNA damage or mutagenic assault and

continue growing in an inflammatory environment characteristic of a tumour. Invasive tumour growth mimics wound healing as neoplastic cells produce an array of cytokines and chemokines to attract leucocytes such as neutrophils, dendritic cells, macrophages, eosinophils, mast cells as well as lymphocytes [37]. Factors that result in chronic inflammation which have been linked to cancer include; *Human Papilloma Virus* infection and cervical cancer, inflammatory bowel disease and colon cancer and *Helicobacter pylori* infection and gastric cancer [38, 39]. Inflammatory components are also present in tumours not originating from inflammatory conditions. Although chronic inflammation may play an important role in the initiation of cancer, post-tumour inflammation has been associated with the maintenance of a pro-tumourigenic microenvironment. When tumours outgrow their nutrient supply they become necrotic in the centre which causes the release of pro-inflammatory mediators to promote neoangiogenesis, cancer cell survival and the recruitment of innate immune cells. The presence of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α have been implicated in promoting tumour progression, migration and invasion [36, 40, 41]. Radiation and chemotherapy have also been known to initiate a tumour-associated inflammatory response. These treatments achieve this by causing necrotic cell death of the tumour cells and surrounding tissue which triggers the body's natural immune response [42]. This reaction has been implicated in the resistance to anticancer treatments [43].

1.5.1 Chemokines and cytokines associated with cancer development

The tumour microenvironment contains various innate immune cells; macrophages, neutrophils, natural killer cells, dendritic cells and mast cells; T and B lymphocytes; cancer cells; cancer-associated fibroblasts and surrounding stromal factors. A network of communication occurs between these cells via the release of cytokines and chemokines or through direct contact to maintain and promote tumour growth and metastasis [34, 44].

Tumour-associated macrophages (TAMs) have an important role in inflammation in neoplastic tissue. They play a dual role in the tumour microenvironment as they can kill tumour cells when activated by IL-2, IFN-γ and IL-12 but also produce a host of potent growth factors, cytokines and proteases that potentiate tumour progression [45]. Tumour cells have the ability to mimic immune cells by producing their own cytokines and chemokines and thereby controlling processes within the tumour microenvironment [46-48]. TAMs and tumour cells produce IL-10 which suppresses the anti-tumour response by cytotoxic T cells [37]. Stromal cells surrounding and integrating with the tumour site play an active role in tumorigenesis and contribute to metastasis by secreting matrix metalloproteases (MMPs). Inflammation at the tumour site is thought to sustain many hallmarks of cancer such as producing growth factors that sustain proliferation, survival factors that limit cells death, proangiogenic components, extracellular modifying enzymes promoting invasion and producing inductive signals which activate the epithelial-to-mesenchymal transition [34, 49]. Transcription factors such as NFkB and AP-1 are key regulators in the expression of chemokines and cytokines. Although an inflammatory response can be initiated in the absence of NFkB this is rarely the case [50].

The following section will discuss the role of key transcription factors, such as NFkB and AP-1, in inflammatory signalling and cancer.

1.6 The Nuclear factor kappa B (NFkB) signalling pathway

Nuclear factor kappa B was first identified as a regulator of κB light chain expression in mature B lymphocytes. Induction of NFkB activity by exogenous stimuli was shown in various cell types through stimulation with lipopolysaccharide (LPS), cyclohexamide and phorbol esters [51, 52]. NFkB is expressed in most cell types and many genes have NFkB binding sites in their promoter or enhancer regions. The broad spectrum of NFkB activity requires tight regulation. Signalling

through the NFkB pathway allows cells to respond to environmental change and is therefore critical for their survival [53]. Pahl (1999) reviewed an extensive list of NFkB activators. Common activators include; cytokines (IL-1, TNF-α), products of infection (LPS, dsRNA), antigen receptors (TCR, BCR), genotoxic stress (UV, γ-radiation) as well as therapeutically used drugs (Cisplatin, Tamoxifen, Doxorubicin) [54].

In mammals the NFkB family consists of five proteins; p65 (RelA), RelB, c-Rel, p105/p50 (NFkB1) and p100/p52 (NFkB2). A combination of these proteins form 15 different homodimeric and heterodimeric complexes of which the p65/p50 heterodimer is the most abundant. Signalling by NFkB occurs through two distinct pathways namely the classical and alternative pathways. The classical pathway results in the activation of the p65/p50 dimer that initiates the transcription of inflammatory mediators and inhibitors of apoptosis. This pathway is activated in an innate immune response by a variety of inflammatory signals including IL-1β and TNF-α which bind to their respective receptors on the surface of the cell. This leads to the recruitment and activation of the IKK complex which then phosphorylates the inhibitor of κB (IκB) leading to its degradation by the proteasome. The p65/p50 subunits are released and transported into the nucleus via specific nuclear importers where it induces transcriptional activation (Fig. 1.3) [23, 55]. IκB interacts with both the p65 and p50 proteins in their dimerized form to mask the nuclear localisation sequence (NLS) thereby causing their cytoplasmic retention [56].

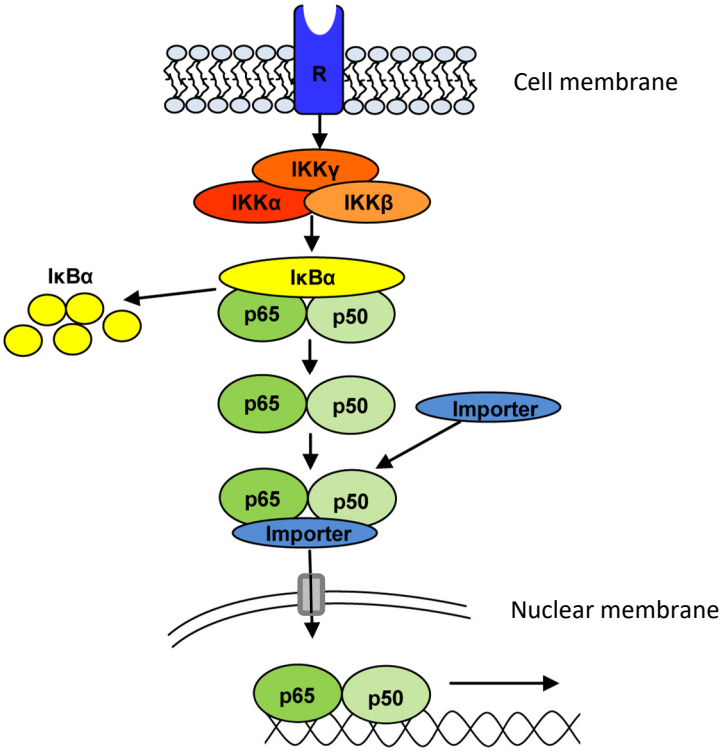


Figure 1.3: The Classical NFkB signaling pathway. The signaling pathway resulting in the activation of the p65/p50 subunits of NFkB.

While the classical NFkB activation pathway is important for inflammatory signalling, the alternative pathway plays a more significant role in the adaptive immune response [55]. This pathway is activated by B-cell activation factor (BAF), CD40 and lymphotoxin β-receptor (LTβR) leading to the activation of IKKα by the NFkB-inducing kinase (NIK). IKKα then phosphorylates p100 leading to the polyubiquitination of p100 and subsequent proteasomal processing to p52. RelB then dimerizes with p52 and together they are transported into the nucleus to initiate transcriptional activation [57]. Apart from the phosphorylation and ubiquitination of IκB and IKK complexes, protein modifications of the subunits which include phosphorylation, ubiquitination and acetylation also play a role in tightly regulating NFkB signalling. Autoregulatory feedback loops also exist as IκBα and p105 are target genes of NFkB. In an unstimulated state NFkB proteins remain inactivated and retained in the cytoplasm however,

constitutively active NFkB has been found in mature B cells, macrophages, neurons, vascular smooth muscle cells as well as in many tumours [53].

1.6.1 NFkB: The link between inflammation and cancer

In addition to the pro- and anti-inflammatory roles of NFkB, there is substantial evidence in the literature suggesting that NFkB has a key tumour-promoting role in cancer. The first clue that NFkB was linked to cancer was apparent with the cloning of *p105/p50* and later *RelA* and the observation of the close relation to the avian viral oncoprotein *v-Rel* and its cellular homolog *c-Rel* [58]. The *p100/p52* gene has also been found to be rearranged in B-cell and T-cell lymphomas which gave rise to a truncated p100 protein devoid of its native IκB-like activity [59]. To date, no mutations in the IκB-NFκB system have been identified in other cancers but rather the increased presence of activated nuclear NFκB has been associated with an increase in inflammation promoting carcinogenesis. The increased activity of NFkB observed in cancer may be due to mutations in genes coding for upstream signalling proteins that feed the NFkB pathway or the exposure to pro-inflammatory stimuli within the tumour microenvironment [57, 60-63].

NFkB is most commonly associated with innate immunity, mediating the inflammatory response. There are however, several other cellular processes where NFkB plays a critical role. It is involved in the promotion of cell proliferation and suppression of cell death. NFkB controls the expression of key cell cycle regulatory genes: cyclin D1, D2 and D3; cyclin E1; c-myc and CDK 2, 4 and 6. It also regulates growth signals like GM-CSF and IL-6 [64, 65]. A study by Jourdan *et al.* (2007) showed that the inhibition of IKKβ resulted in a block in cell cycle progression in multiple myeloma [66]. While NFkB also induces expression of genes encoding anti-apoptotic molecules thereby supressing cell death pathways and further promoting

growth [67]. Resistance to apoptosis in human cancer cell lines is potentially linked to activation of NFkB because subsequent inhibition of NFkB allowed apoptosis to be triggered more easily [64]. Failure of chemotherapeutics has been associated with NFkB-induced apoptotic resistance [68]. The progression of localised tumours to metastatic cancer involves changes in the relationship between cancer cells and the extracellular matrix favouring migration and invasion. Classical NFkB activation has been implicated in epithelial-to-mesenchymal transition (EMT), a process leading to invasion and metastasis. Inhibition of NFkB was able to reverse EMT in mesenchymal cells [69]. NFkB is also implicated in metastasis through the expression of various matrix-modifying enzymes (MMP-2/-9) and adhesion proteins (VCAM-1, ICAM-1) [70-73]. As the tumour expands and the demand for nutritional resources outweigh the supply, new blood vessels are required. Angiogenesis is largely triggered by NFkB signalling. NFkB inflammatory mediators and target genes; TNF-α, IL-1 and IL-6 can stimulate the expression of one of the main regulators of angiogenesis, vascular endothelial growth factor (VEGF). VEGF also happens to be a target gene of NFkB [64]. Given the broad range of cellular processes in which NFkB is involved it is not surprising that aberrant NFkB signalling is associated with carcinogenesis. Tumours observed to have high levels of p65 include; breast and stomach adenocarcinoma, non-small cell lung cancer, multiple myeloma and B-cell non-Hodgkin's lymphoma. High levels of p50 has been observed in; non-small cell lung cancer and colon, breast, prostate, bone and brain cancer cell lines [23].

Having previously discussed the causal link between inflammation and cancer and given the observed activated levels of NFkB in many cancers it was proposed that NFkB may be the missing link between inflammation and cancer [74].

1.6.2 Targeting NFkB for cancer therapy

The diverse and essential role that NFkB plays in the maintenance of several of the hallmarks of cancer make it an attractive anticancer target. When designing an NFkB inhibitor the role of this transcription factor in normal cells and in innate immunity should be taken into consideration. The ideal inhibitor should be more active in cancer cells than normal cells and the effect should be transient or even reversible to avoid extended immunosuppression. Seven possible points of interception within the NFkB signalling pathway have been reviewed by Gupta *et al.* (2010). These points of interception include; 1) Inhibition of protein kinases within the IKK complex thereby inhibiting IκBα phosphorylation. 2) Inhibition by protein phosphatases which act as a counterbalance inhibiting kinase action. 3) Proteasome inhibition and IκB ubiquitination blockers as the ubiquitination and subsequent degradation of IκBα is an essential step for releasing NFkB from its cytoplasmic inhibitor. 4) Blocking nuclear translocation of NFkB as it is essential for it to be in the nucleus to bind the DNA. 5) Blocking NFkB activation by inhibiting p65 acetylation which occurs in the nucleus prior to DNA binding and is required for activation. 6) Blocking NFkB activation by methyltransferase as methylation of p65 acts as a negative regulator by causing the destruction of DNA-bound, active NFkB and lastly 7), the most direct strategy would be to block the transcription factor from binding the DNA sites required for target gene transcription [75].

Intercepting the NFkB signalling pathway at the point of nuclear translocation of the transcription factor is of particular interest to our work. Nuclear import is mediated by the Karyopherin proteins. Detail on the nuclear transport system is described in section 1.8 below. Briefly, Karyopherin beta 1 (KPNB1), a nuclear import protein that works in unison or together with one of the 6 Karyopherin alpha (KPNA) adaptor proteins to transport proteins containing a nuclear localisation sequence (NLS) into the nucleus. NFkB proteins have been reported to

contain a NLS that is recognised by Karyopherin proteins. A series of peptides have been designed to mimic the NLS of p50/NFkB thereby interfering with the nuclear translocation of NFkB. The peptide, SN50, was able to target the classical NFkB signalling pathway inhibiting the translocation of NFkB into the nucleus of endothelial and monocytic cells [76]. The N50 motif was found to interact with Karyopherin alphas during stimulus-induced signalling thereby inhibiting the nuclear import of NFkB. A study by Zienkiewicz *et al.* (1999), reported the N50 peptide preferentially binds KPNA1. KPNA1 has been shown to have a more significant role in importing the STAT1 transcription factor [77]. Despite this, SN50 is still a commonly used peptide for inhibition of NFkB in laboratory-based research [78, 79]. A similar peptide, SN52, blocked the nuclear import of the RelB:p52 dimer thereby targeting the alternative NFkB pathway without affecting the classical pathway [80]. A peptide also designed to mimic the p50 NLS and inhibit NFkB activity, likely similar to SN50, was found to reduce inflammation and oxidative stress in vascular cells and macrophages as well as protect mice from the development of atherosclerosis [81]. Theiss *et al.* (2009) found that overexpressing prohibitin 1 in intestinal epithelial cells suppressed the expression of KPNA4 thereby inhibiting NFkB translocation [82]. There is conflicting evidence in the literature regarding which nuclear import proteins are responsible for transporting NFkB into the nucleus in the classical signalling pathway. Fagerlund *et al.* (2008) and Theiss *et al.* (2009) report that nuclear import of NFkB is mediated by KPNA3 and KPNA4 while Liang *et al.* (2013) report that the KPNB1/KPNA2 transport system is needed for NFKB transport [82-84].

Several studies have reported that nuclear import inhibition of NFkB has anti-inflammatory effects in normal cells but far fewer have shown these effects in cancer cells. Small molecule inhibitors are classically designed to investigate the role of a particular protein in a disease condition. Newer small molecule inhibitors of NFkB include; dehydroxymethyl derivatives of epoxyquinomicin C. These (-)-DHMEQ compounds selectively inhibit NFkB by binding to it and

inhibiting its DNA binding activity and has also been found to inhibit its nuclear translocation. Even though these compounds have shown anti-inflammatory and anticancer activity in mice they have so far not been developed further for clinical use [85, 86].

Designing new drugs, testing them and acquiring FDA approval can be a very lengthy process. This together with the fact that the mechanism of actions of many approved drugs are not fully understood, has led scientists to re-evaluate and repurpose already FDA-approved drugs. Several known drugs have been found to have unappreciated inhibitory effects on NFkB. Drugs such as emetine, fluorosalan, sunitinib malate, bithionol, narasin, tribromsalan, and lestaurtinib were found to inhibit NFkB signalling through inhibition of IκBα phosphorylation and cause cell death in cervical cancer cells. While the cardiac glycosides, digitoxin and ouabain, inhibited NFkB activity and were potent inhibitors of cancer cell growth [87]. The anticancer effect of these drugs could well in part be attributed to their inhibitory effect on NFkB. Further research is required to design effective NFkB inhibitors, particularly ones that preferentially affect cancer cells.

1.7 The Activating Protein 1 (AP-1) signalling pathway

Activating Protein 1 (AP-1) is a transcription factor comprising of JUN, FOS, Activating Transcription Factor (ATF) and Musculoaponeurotic Fibrosarcoma (MAF) protein families that function in a dimeric complex. The combination of homo- and heterodimers constitutes functional AP-1. AP-1 most commonly consists of c-JUN and c-FOS homo- and heterodimers [88, 89]. Much like NFkB it is activated by inflammatory cytokines and growth factors as well as the phorbol ester, PMA [90]. Activation of AP-1 occurs through stimulation of the Mitogen Activated Protein Kinase (MAPK) pathway leading to phosphorylation of an AP-1 subunit allowing its dimerization and DNA-binding [91, 92]. Phosphorylation of the c-JUN component

of AP-1 occurs at the serine 63 and serine 73 residues of the protein [93]. The c-JUN N-terminal Kinase (JNK) most commonly performs this phosphorylation [94]. Once the subunits have been phosphorylated and dimerised they bind their respective DNA-binding sites and initiate transcription of various target genes. AP-1 target genes are involved in many cellular processes including; proliferation, differentiation, apoptosis, angiogenesis and tumour invasion [89, 95, 96]. The diverse role of AP-1 is also attributed to its interactions with other transcription factors such as; NFkB, NFAT, Sp1 and Smad [97-101].

1.7.1 Altered AP-1 expression and activity associates with multiple aspects of cancer development

The diverse role that AP-1 plays in normal cellular functioning it is not surprising given that increased expression of JUN and FOS family members is associated with several cancers [88, 96, 102]. The c-JUN and c-FOS subunits have long been characterised as oncogenes by their homology to the Finkel-Biskis-Jinkins viral counterparts; v-JUN and v-FOS [103]. Upregulation of these proteins through overexpression or by RAS signalling pathways is well established in promoting transformation leading to tumourigenesis [88]. Many of the oncogenic properties manifest through the promotion of the “Hallmarks of Cancer”.

AP-1 plays an important role in cell proliferation and survival primarily through the regulation of target genes; cyclin D1, p53, p21 and p16. Cell cycle progression is promoted when c-JUN is activated by JNKs leading to the expression of cyclin D1. While JunB and JunD have been reported to have anti-proliferative affects their expression is often repressed in highly proliferative tumours [89]. Similarly, the AP-1 family members can also have contrasting pro-apoptotic and anti-apoptotic roles which could be mediated by the differential expression of pro- and anti-apoptotic target genes. c-JUN also plays a repressive role on the tumour

suppressor, p53, which in turn promotes survival. The effects of c-JUN on survival reported to be cell-specific [88, 96].

Inflammation and angiogenesis have previously been established as critical components of cancer initiation and progression. Working synergistically with the transcription factor, NFkB, AP-1 can enhance the inflammatory response through the expression of cytokines; IL-6, IL-1 and TNF-α [42, 97]. Several target genes of AP-1 have also been implicated in angiogenesis. c-FOS was found to upregulate the vascular endothelial growth factor family member, VEGF-D. The FRA1 subunit upregulates urokinase-type plasminogen activator (uPA) and its receptor (uPAR) and c-JUN and JunB were found to upregulate proliferin [88].

Invasive tumour growth requires degradation of the extracellular matrix (ECM), EMT and migration of cellular components all of which require genes regulated by AP-1. Matrix metalloproteases are responsible for the degradation of the ECM and many have AP-1 binding sites including; MMP-1, MMP-3 and MMP-9. Inhibition of AP-1-mediated MMP-9 expression has been reported to inhibit the invasive ability of liver and breast cancer cell lines [88, 104-106]. Both c-JUN and c-FOS are able to induce EMT by causing the loss of cell polarity [107, 108]. The combined contribution of AP-1 in enabling the hallmarks of cancer makes it of interest as a transcription factor to target in cancer therapy.

1.7.2 Targeting AP-1 for cancer therapy

The MAPK activation pathway of AP-1 provides several opportunities to target AP-1 activity. However, given the diverse role of MAPK signalling proteins, inhibiting MAPK proteins may lead to effects not specifically related to AP-1. Few studies have focused on targeting AP-1 directly. A dominant negative mutant of c-JUN, TAM67, that lacks the transactivation domain

but can dimerise with other AP-1 members and bind the DNA, has been shown to inhibit AP-1 activity. TAM67 has been reported to reduce transformation and tumourigenesis [22, 109].

To identify specific inhibitors of AP-1, Ruocco *et al.* (2007) used a high throughput cell-based assay. They screened libraries of synthetic compounds, pure natural products and natural product extracts. These authors report that geldanamycins, heat shock protein 90 inhibitors, could reduce c-JUN expression and JNK activation. While bryostatins, reserpines and nigericins reduced AP-1 activation through the modulation of protein kinase C [110]. Natural compounds such as dihydroguaiaretic acid and curcumin have been reported to directly target AP-1 by interfering with DNA binding of the c-JUN:c-FOS dimer. This inhibition had cytotoxic effects on leukemia, lung cancer and colon cancer cells *in vitro* [111]. Depending on the point of interception some compounds targeting signalling pathways resulting in the activation of specific transcription factors may be able to target more than one transcription factor simultaneously. The NFkB inhibitors of the SN50 family have also shown inhibitory effects on the nuclear localisation and activity of AP-1 [112, 113]. Finding new strategies to directly target the AP-1 oncogenes, c-JUN and c-FOS, could be beneficial as an anticancer approach. One such strategy is to target the nuclear import of these oncogenes. Nuclear import of the AP-1 subunit c-JUN has been suggested to be mediated through the non-classical nuclear import pathway, which functions independently of KPNA adaptor proteins. KPNB1 was found to have a significantly greater affinity for AP-1 than Karyopherin alpha and therefore is reported to be transported into the nucleus by KPNB1 alone [114].

1.8 The Karyopherin family of nuclear transport proteins*

The Karyopherin superfamily is a major class of soluble nuclear transport proteins consisting of both import and export proteins. The trafficking of proteins in and out of the nucleus is essential for proper cellular functioning and cell survival. Proteins greater than 20-40kDa in size are unable to enter the nucleus through the nuclear pore complex (NPC) unaided and rely on facilitated transport. This facilitated transport of numerous proteins is achieved by the action of Karyopherin proteins. The larger Karyopherin superfamily is subdivided into the Karyopherin beta/Importin beta family and the Karyopherin alpha/Importin alpha family. They function together to achieve the nuclear transport requirements of cargo proteins. To date, 20 genes encoding Karyopherin beta proteins in the human genome have been identified. Ten are nuclear import proteins, seven are involved in nuclear export, two bidirectional transporters and one protein that is yet to be characterised (Table 1.1) [115].

* Sections 1.8 and 1.9 are adapted from work published as a review in IUBMB Life (Stelma *et al.* 2016)

Table 1.1: Classification of nuclear transporters and their association to cancer

Nuclear Transporters	Symbol	Synonyms	Associated with cancer
A) Import			
Karyopherin beta 1	KPNB1	NTF97, IPOB, MGC2155, MGC2156, MGC2157, IMB1, Impnb, IPO1	YES
Transportin 1	TNPO1	KPNB2, MIP, TRN, IPO2, MIP1	-
Transportin 2	TNPO2	IPO3, KPNB2B, FLJ12155, TRN2	-
Transportin 3	TNPO3	LGMD1F, TRN-SR, MTR10A, TRN-SR2, IPO12	-
Importin 4	IPO4	Imp4, FLJ23338	-
Importin 5	IPO5	KPNB3, RANBP5, IMB3, MGC2068, Pse1	-
Importin 7	IPO7	RANBP7, Imp7	-
Importin 8	IPO8	RANBP8, IMP8	-
Importin 9	IPO9	Imp9, FLJ10402	-
Importin 11	IPO11	RanBP11	-
Karyopherin alpha 1	KPNA1	SRP1, RCH2, NPI-1, IPOA5	-
Karyopherin alpha 2	KPNA2	RCH1, SRP1alpha, IPOA1, QIP2	YES
Karyopherin alpha 3	KPNA3	SRP1gamma, SRP4, hSRP1, IPOA4	-
Karyopherin alpha 4	KPNA4	QIP1, SRP3, IPOA3, MGC12217, MGC26703	YES
Karyopherin alpha 5	KPNA5	SRP6, IPOA6	-
Karyopherin alpha 6	KPNA6	IPOA7, KPNA7, MGC17918, FLJ11249	-
Karyopherin alpha 7	KPNA7	IPOA2	YES
B) Export			
Exportin 1	XPO1	CRM1, emb	YES
CSE1 Chromosome Segregation 1-Like (Yeast)	CSE1L	CAS, XPO2, CSE1	YES
Exportin 5	XPO5	KIAA1291	YES
Exportin 6	XPO6	RANBP20, KIAA0370, FLJ22519	-
Exportin 7	XPO7	RANBP16, KIAA0745	-
Exportin t	XPOT	XPO3	-
Ran binding protein 17	RANBP17		-
C) Bidirectional			
Exportin 4	XPO4	FLJ13046, KIAA1721	YES
Importin 13	IPO13	IMP13, KIAA0724, RANBP13	-
D) Uncharacterised			
Ran binding protein 6	RANBP6		-

Nuclear import of cargo proteins is most commonly described by the Classical and Non-Classical import pathways (Fig. 1.4).

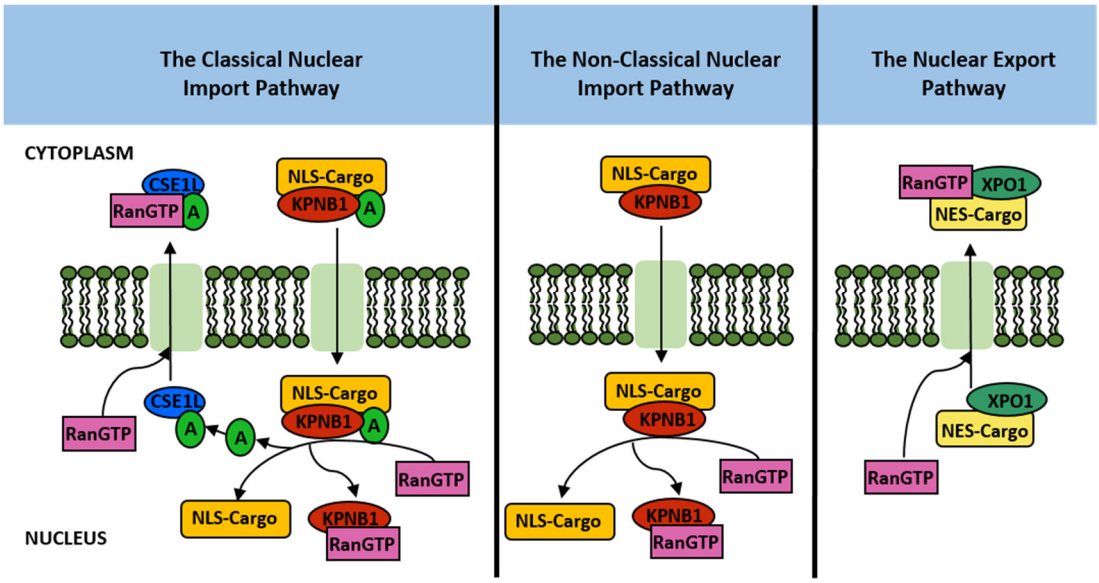


Figure 1.4: The Nuclear Import and Export pathways. In the Classical nuclear import pathway NLS-cargo is transported into the nucleus by KPNB1 and an adaptor protein, Karyopherin alpha (A). The nuclear transporters and cargo dissociate in the nucleus upon RanGTP binding. Karyopherin alpha is transported back into the cytoplasm by the export protein, CSE1L. The Non-Classical nuclear import pathway transports NLS-cargo into the nucleus using KPNB1 alone. The nuclear export pathway transports NES-cargo from the nucleus into the cytoplasm stabilised by Ran GTP.

The Classical import pathway involves a Karyopherin alpha adaptor protein that recognises and binds a cargo protein containing a classical nuclear localisation sequence (NLS). Cargoes transported by the classical import pathway include STAT family members and GATA-3 [97]. The KPNA/cargo dimer is then bound by Karyopherin beta 1 (KPNB1) to form a trimeric complex that can pass through the NPC. The Non-Classical import pathway on the other hand bypasses the need for an adapter protein and the cargoes bind KPNB1 directly. A few unique cargo proteins such as sterol regulatory-related protein (SREPB2) and parathyroid hormone-related protein (PTHrP) use this Non-Classical method of nuclear import [116]. Passage through the NPC is mediated by the interactions between Phe-Gly repeat-containing

nucleoporins of the NPC and KPNB1. A key event, upon arrival in the nucleus, is the binding of RanGTP to the KPN/cargo complex promoting the dissociation of the import complex, allowing the cargo to remain in the nucleus and the transport proteins to be recycled [117, 118]. The nuclear export protein, CSE1L, is responsible for recycling the Karyopherin alphas back into the cytoplasm. Nuclear export of protein cargo is primarily achieved through Exportin 1 (XPO1) recognising a leucine-rich nuclear export signal (NES) on the cargo and binding it to it. RanGTP stabilises the XPO-1 bound NES-cargo complex that can then pass through the NPC towards the cytoplasm. The hydrolysis of RanGTP to RanGDP, in the cytoplasm, allows the dissociation of the export protein and the cargo (Fig. 1.4) [119]. Although the best described role of Karyopherin proteins is the nuclear import and export of cargo proteins they also play an important role in mitosis and other cell cycle-regulated functions including regulating the assembly of the NPC and nuclear membrane at mitotic exit as well as DNA replication at S-phase [120, 121].

1.9 Nuclear transport in cancer

The Karyopherin proteins play a fundamental role in normal cell physiology by providing the means of trafficking between the cytoplasm and the nucleus as well as in other cellular processes such as mitosis. As intracellular localisation of many proteins, as well as the nuclear transporters themselves has an impact on their activity status and/or function, the correct spatial arrangement of proteins at the correct time is critical. It is therefore no surprise that impaired regulation of nuclear-cytoplasmic transport has been reported to associate with pathogenesis. A well-researched area is the association of nuclear transport dysregulation and cancer, which most commonly leads to the mislocalisation of cancer-associated cargo proteins. For example, the nuclear accumulation of the DNA repair protein, NBS1, has been shown to correlate with gastric cancer progression while the cytoplasmic accumulation of

Wilms' Tumour protein, Wt1, was observed more frequently in malignant tissues than normal [122, 123].

Dysregulated activity of a number of Karyopherin proteins have been implicated in cancer (Table 1.1, [124-132]). This literature review will focus on KPNB1/KPNA2-mediated nuclear import and XPO1-mediated nuclear export. There are many junctions in the nuclear transport pathways at which dysregulation can occur that have been reported to be linked to cancer. These forms of dysregulation include; the altered expression of the nuclear transporters, altered localisation of nuclear transporters and the mechanistic implications of nuclear transporters in mitotic division and genetic instability.

1.9.1 Dysregulation of nuclear transporters

Elevated expression of Karyopherin proteins that associates with the global dysregulation of protein transport has been observed in various types of cancer. Amongst all members of the Karyopherin family; XPO1, KPNB1 and KPNA2 are the most frequently reported to be overexpressed in cancer. Kuusisto *et al.* (2012) found that the increased expression of these Karyopherin proteins in transformed cells correlates with enhanced nuclear import and export efficiencies in transformed cells [133]. The increased expression and thus transport of cargos across the nuclear membrane is proposed to be a mechanism allowing cancer cells to cope with their increased metabolic and proliferative demands. The increased expression of import proteins, in particular, might allow for increased nuclear entry of proteins that have oncogenic tumour promoting functions, for example ERK1/2, c-Myc and E2F1 [134-136].

The link between Karyopherin overexpression and cancer has been the subject of numerous studies however, few studies have addressed the underlying mechanism leading to their

overexpression. Using molecular and bioinformatics approaches, research in our laboratory showed that the overexpression of both KPNB1 and KPNA2 is primarily due to dysregulated E2F/Rb activity in cancer cells [129]. The constitutive activation of E2F in e.g. cervical cancer cells was found to associate with increased expression of both KPNB1 and KPNA2. It is well-known that HPV infection in cervical cancer results in E2F dysregulation and thus elevated KPNB1 and KPNA2 in this cancer. The E2F/Rb pathway is disrupted in a remarkably high proportion of human cancers through other mechanisms leading to the same overexpression phenotype [137]. Interestingly, Kuusisto *et al*. (2015) showed that the extent of overexpression of KPNB1 correlates with disease state in the MCF10 human breast tumour progression system, suggesting that its overexpression not only correlates with E2F dysregulation, but can vary according to tumour progression state [138]. Other mechanisms for altered Karyopherin expression have been reported. A recent study showed elevated KPNA2 expression resulting from decreased expression of microRNA-26b in epithelial ovarian carcinoma. MicroRNA-26b directly targets KPNA2 by repressing its translation and thus reduced microRNA-26b resulted in elevated KPNA2 expression [139].

Quan *et al.* (2008) used bioinformatic approaches to predict transcription factors that bind the promoter regions of Karyopherin beta genes and identified Sp1, NRF-2, HEN-1, RREB-1 and NFY as potential regulators of Karyopherin beta expression [140]. In line with this, van der Watt *et al*. (2011) found that NFY and Sp1 are both potential contributors to XPO1 overexpression in cervical cancer and transformed cells [129]. A study by Kahle *et al.* (2005), found that NFY-A is imported into the nucleus by KPNB1 where it acts to transcribe its downstream targets [141]. This finding could explain why elevated expression of KPNB1 has been observed concomitant with elevated XPO1 in cervical and gastric cancer [128, 130, 142]. van der Watt *et al.* (2009) also showed that under DNA damage conditions the elevated p53 level plays a repressive role in the transcription of the *xpo1* gene [128]. It is not surprising then

that XPO1 overexpression is commonly observed in cancer as p53 is often expressed at low levels or mutated in many cancers [143]. XPO1 has also been shown to be involved in p53 nuclear export [128] suggesting that a possible feed-back regulatory mechanism may exist. Under DNA damage conditions p53 expression increases and enters the nucleus thereby repressing XPO1 expression. The reduced XPO1 expression will in turn enhance p53 retention in the nucleus further repressing XPO1 expression. In p53 deficient or mutant cells the repression of XPO1 expression is relieved, likely leading to XPO1 overexpression.

The ability of nuclear transporters to efficiently execute their functions depends on their ability to interact correctly with other members of the transport cycle. A truncated KPNA lacking part of the cargo-NLS binding domain has also shown failure to interact with and transport p53 into the nucleus in breast cancer cells. Despite the KPNB1 binding domain remaining intact in the mutant KPNA, it was localized predominantly in the cytoplasm and perinuclear region as opposed to the even distribution observed in wildtype KPNA [144]. This suggests that cargo recognition and binding by KPNA is a prerequisite for nuclear entry of proteins such as p53, and that pairing with KPNB1 alone is not sufficient.

When nucleo-cytoplasmic transport ceases, virtually all transport factors take on new roles in mitosis, acting in the functional organisation of the mitotic spindle and in the reconstitution of the interphase nucleus at mitotic exit. Modelling studies have indicated that subtle dysregulation of the expression of transport factors readily affects mitotic division and can cause significant abnormalities in chromosome segregation, whereas nucleo-cytoplasmic transport is a more robust process [145, 146]. Dysregulation of nuclear transport factors can therefore cause the onset of genetic instability, a cancer hallmark.

The role of KPNB1 as a regulator of spindle formation and function is well described. KPNB1 generally acts by preventing the premature localisation or activity of spindle regulatory factors [147]. Factors, with spindle assembly functions, regulated by KPNB1 include; the spindle pole-organizing protein NuMA [148], the microtubule-regulatory protein HURP [149], Rae1/Nup98 [150], a regulator of the Aurora-A kinase maskin [151] and APC [152] among others. KPNB1's physical association and regulation of the *Adenomatous polyposis coli* (*Apc*) oncogene, itself a microtubule-promoting and mitotic spindle-associated factor, may be of particular relevance to the onset of colon cancer [152]. A novel function through which KPNB1 can globally regulate the mitotic apparatus has recently emerged and relates to its ability to regulate, in concert with the APC/C ubiquitin ligase, the stability of mitotic spindle regulatory factors [153]. Karyopherin alpha family members although difficult to discuss collectively, share a common functional feature that their down-regulation induces substantial levels of apoptosis [154]. They contribute to mitotic control by interacting with key spindle regulatory factors such as TPX2 [155].

The nuclear export protein, XPO1, has well defined mitotic effects exerted at various levels of mitosis. These include the mitotic spindle [156]; kinetochores, stabilizing microtubule/kinetochore interactions essential for chromosome segregation [157]; and centrosomes, preventing chromosome reduplication which constitutes a major threat to genome stability [158, 159]. These effects are mediated through several mitotic targets that contain NES sequences. The interplay between XPO1 and mitotic cyclin B is interesting to note. XPO1 contributes to cyclin B localization and in turn cyclin B-CDK 1 complex phosphorylates XPO1 to finely regulate its mitotic functions [160]. It has also been reported that XPO1's interaction with survivin is important to target the chromosomal passenger protein to kinetochores [161].

Nuclear transport plays a critical role in the functioning of many cellular processes. Because of this, cells have developed systems to tightly control the nuclear transport processes. Within each junction in the regulatory pathway lies an opportunity for errors to occur, and indeed many of these alterations have been associated with cancer.

1.9.2 Nuclear transporters as potential diagnostic markers of cancer

Late diagnosis of cancer is a major contributing factor to poor patient outcome. Often, by the time patients present at clinics, the cancer has already progressed to later stages and the chances of the primary tumour having already metastasised is relatively high. This highlights a need for effective tools for the early detection of cancer. The ideal biomarker would be unique to cancer and present in easily obtainable patient samples such as serum or urine. Nuclear transporters have already been shown to be upregulated at the protein level in many cancer tissues and a few studies have investigated these proteins as potential diagnostic biomarkers. Wang *et al.* (2011) investigated potential biomarker targets that were both significantly up-regulated in lung cancer tissues and secreted/released from lung cancer cells. They achieved this by integrating two lung adenocarcinoma cell line secretome datasets with one adenocarcinoma microarray dataset. Using this strategy they identified KPNA2 as a potential diagnostic biomarker for adenocarcinoma. Elevated KPNA2 serum levels were confirmed in a cohort of non-small cell lung carcinoma patients (n=126) in comparison to healthy individuals (n=64) [162]. Similar to what was observed in lung cancer patients, KPNA2 levels in serum were also significantly upregulated in oesophageal squamous cell carcinoma, epithelial ovarian cancer and colorectal cancer patients versus healthy controls validating the potential of KPNA2 as a diagnostic biomarker[163-165]. KPNA2 has also been identified as a potential diagnostic biomarker that can differentiate between grades of astrocytoma. This however, still requires immunohistochemical validation. The conventional criteria for

differentiating between WHO grade II and III astrocytomas is particularly difficult to define. Gousias *et al.* (2012) found that patients diagnosed with WHO grade II astrocytoma showed little/no nuclear KPNA2 immunostaining whereas half of the patients diagnosed with anaplastic astrocytoma (WHO grade III) showed ≥5% KPNA2 staining in the nucleus [166].

A recent study in our laboratory, using mass spectrometry analysis of proteins secreted in exosomes by cervical and oesophageal cancer cells found KPNB1 and XPO1, among other nuclear transport proteins, to be secreted at elevated levels compared to normal cells (unpublished data; A.Wishart, MSc Dissertation, 2017)[†]. It is thus possible that these proteins might have potential as diagnostic markers.

1.9.3 Nuclear transporters and patient prognosis

A study by van der Watt *et al.* (2009) found that KPNB1, KPNA2 and XPO1 are all upregulated in cervical cancer patient samples in comparison to normal tissue. KPNB1 and XPO1 but not KPNA2 were found to be essential for the survival of cervical cancer cells [128]. The nuclear importer, KPNB1, and exporter, XPO1, both show promise as cancer biomarkers and studies aimed at investigating their potential as prognostic markers for cervical cancer are underway. More recently Zhu *et al.* (2015) found that KPNB1 was upregulated in gastric cancer patient tissue and cells in comparison to their normal counterparts. Zhou *et al.* (2013) found XPO1 protein levels to be significantly upregulated in gastric cancer patient tissues as well, indicating that both import and export machinery is upregulated in gastric cancer. The upregulation of KPNB1 correlated positively with Ki-67 immunostaining, infiltration depth and tumour grade but not TNM stage and lymph node metastasis. On the other hand, overexpression of XPO1

[†] A. Wishart. "Investigating secreted biomarkers for cancer: The potential of nucleotransport proteins." MSc dissertation, University of Cape Town, 2017.

positively correlated with TNM stage as well as metastasis. Both increased KPNB1 and XPO1 expression have been identified as independent prognostic factors to predict gastric cancer patient survival [130, 142].

XPO1 has also been identified as a suitable independent prognostic marker in ovarian cancer, pancreatic cancer, osteosarcoma, brain cancer and acute myeloid leukaemia [167-172]. Studies on pancreatic cancer as well as osteosarcoma revealed XPO1 overexpression to be associated with increased tumour size as well as histological grade in osteosarcoma but not pancreatic cancer. For both pancreatic cancer and osteosarcoma there is evidence suggesting that increased XPO1 expression is an indicator of reduced overall and progression-free survival [168, 171]. Although expression levels of nuclear transporters often enhance their prognostic value, in some cases so does their localisation within the cell. While the localisation of XPO1 between the nucleus and the cytoplasm showed no significant differences among gastric cancer samples, the cellular localisation of XPO1 in ovarian cancer has been correlated with different aspects of cancer progression [130]. Enhanced cytoplasmic XPO1 has been associated with advanced ovarian tumour stage, poor differentiation and higher mitotic rate. Nuclear XPO1 levels have been associated with enhanced Cox-2 expression leading to poor overall patient survival [169]. Nuclear XPO1 has also been correlated with pathological stage in gliomas [170]. XPO1 localisation is also altered during oesophageal tumourigenesis, where it shifts from predominantly nuclear in normal tissue, to nuclear and cytoplasmic in cancer tissue. This appears to occur in the early stages of disease progression [173].

The potential of nuclear transporters such as KPNB1 and XPO1 as prognostic tools is still under investigation and data supporting this is only available for a select few cancers. KPNA2 however, has been well established as a prognostic marker in various cancers including; breast cancer, brain cancer, gastric cancer, prostate cancer, ovarian cancer, bladder cancer, liver

cancer, colorectal cancer, melanoma, lung cancer and oesophageal cancer [122, 165, 167, 174-179]. Dahl *et al.*'s (2006) research on breast cancer was the first to identify KPNA2 as a prognostic marker [180]. Since then high nuclear KPNA2 expression has been linked to poor patient outcome. Numerous studies have shown that KPNA2 expression correlates with overall and progression-free survival in patients and established KPNA2 as an independent prognostic marker when compared to other clinical data [122, 166, 167, 176, 178, 179, 181-183]. Interestingly, KPNA2 expression also predicted the chances of metastasis as well as histological grade and clinical stage of tumours. High KPNA2 expression in non-invasive bladder cancer increased the risk of progression to a more invasive form [182].

Some malignancies have a high recurrence rate following therapeutic or surgical intervention and it is important to identify risk factors in these patients that might predict recurrence. KPNA2 expression has been identified as a marker of both early and more frequent recurrence in liver cancer while it is also a marker of PSA recurrence in prostate cancer [177, 184, 185]. In meningioma, elevated KPNA2 and XPO1 expression was observed in recurrent tumours in comparison to primary tumours and this correlated with increased recurrence rates [167].

The evidence presented here highlights that KPNA2 serves as a valuable prognostic marker throughout the progression of cancer from early stage recurrence to chances of metastasis, as well as overall survival. While there is evidence that KPNB1 and XPO1 have shown potential as prognostic markers, this requires further investigation and validation. Evidence presented in a study by Gousias *et al.* (2014), shows KPNA2 expression levels correlating with XPO1 expression levels in meningioma, suggesting that multiple nuclear transporters may too have prognostic value in cancer [167].

1.9.4 Potential of nuclear transporters as chemotherapeutic targets

The upregulation of nuclear transporters and their association with poor prognosis in cancer highlights their potential as therapeutic targets. Challenges may arise though when targeting cellular machinery that is active in both normal and cancer cells. However, studies have shown that cancer cells are more sensitive to nuclear transport inhibition than non-cancer [128, 138, 186]. Specific knockdown using siRNA for nuclear import proteins, KPNB1 and KPNA2, and export protein, XPO1, in cancer cells frequently results in reduced proliferation and increased apoptosis [135, 154, 183, 185, 187]. This suggests that cancer cells may have an enhanced dependence on nuclear transporters for their increased proliferative and metabolic demands (referred to as tumour cell "addiction").

1.9.4.1 Nuclear export inhibitors as anticancers

Among the Karyopherin family members the nuclear exporter, XPO1, has so far been the most successful chemotherapeutic target already being tested in clinical trials (Table 1.2). Several natural products were first described as inhibitors of XPO1 with Leptomycin B being the most potent. Leptomycin B showed severe cytotoxic effects in phase I clinical trials and was no longer pursued for use in patients. Subsequently, several Leptomycin B derivatives such as Anguinomycin and Kos-2464 have been synthesized in an attempt to reduce the cytotoxity while retaining the ability to inhibit nuclear export via XPO1, but have as yet not entered clinical trials. A recent review by Ishizawa *et al.* (2015) outlines the history of XPO1 as a chemotherapeutic target in more detail [188]. In 2012 the SINE (Selective inhibitors of nuclear export) series of drugs were identified through an *in silico* molecular modelling strategy and synthesized by Karyopharm Therapeutics (Karyopharm Therapeutics, Inc., Boston, MA, USA) [189]. These compounds are water-soluble and irreversibly modify a cysteine (Cys528) in the

NES-binding groove of XPO1 thereby inhibiting the function of the protein. KPT-185 is the most potent of the series and is most commonly studied *in vitro.* However KPT-330/Selinexor, while nearly as potent, has been reported to have more acceptable pharmacokinetics and has shown promise in phase I, II and III clinical trials in both haematological and solid tumours [190-192].

Table 1.2: Nuclear transport inhibitors

Nuclear transporter	Inhibitor	Nature of the compound	Experimental Status
KPNB1	cSN50.1 [77]	Peptide	Cell Culture
KPNA/B1	Ivermectin [193]	Antibiotic	
	cSN50.1 [77]	Peptide	Cell Culture
	Bimax1 [112]	Peptide	Cell Culture
	Bimax2 [112]	Peptide	Cell Culture
	Karyostatin 1A [194]	Small molecule	Cell Culture
	Importazole [195]	Small molecule	Cell Culture
XPO1	Leptomycin B [196]	Antibiotic	Phase I Clinical Trials (discontinued)
	Ratjadone analogs [188]	Antibiotic	Cell Culture
	Anguinomycin [188]	Antibiotic	Cell Culture
	Goniothalamin [188]	Organic molecule	Cell Culture
	Kos-2464 [188]	Small molecule	Xenograft Mouse Model
	N-azolylacrylate analogs [188]	Small molecule	HIV Model
	CBS9106 [188]	Small molecule	Xenograft Mouse Model
	SINE series [189]	Small molecule	Phase I-III Clinical Trials

1.9.4.2 Potential of nuclear export inhibitors in combination therapies

More recently combination treatment strategies using XPO1-inhibitors and currently available chemotherapeutic agents have proven to be effective in enhancing the treatment of cancer in model systems. Nuclear export inhibitors alone, while effective in causing cell death *in vitro,* in animal models these inhibitors while inhibiting tumour growth were less often likely to eliminate existing tumours. The combination therapy is thought to use the XPO1-inhibitor to sensitise cancer cells to chemotherapeutic agents. A review by Turner *et al.* (2014) extensively discusses a range of studies providing evidence for the efficacy of XPO1-inhbitors in combination with alkylating agents, anthracyclines, BRAF inhibitors, platinum drugs, protease inhibitors and tyrosine-kinase inhibitors against both haematological and solid tumours [197]. SINE compounds; KPT-185, KPT-249 and KPT-330 were able to sensitize myeloma cells to doxorubicin, bortezomib and carfilzomib and phase I/II clinical are currently underway [197, 198]. A combination therapy using KPT-330 and gemcitabine has also been found to synergistically enhance cell death *in vitro* and *in vivo* in pancreatic cancer [199].

1.9.4.3 Nuclear import inhibitors as anticancers

The field of nuclear import inhibitors, while not as advanced as that of nuclear export inhibitors at present, is growing (Table 1.2). Most commonly KPNB1 and the KPNA isoforms work together to transport cargoes into the nucleus, although KPNB1 can also transport cargo independently. Therefore, targeting KPNB1 may have a broader spectrum of import inhibition while targeting a single KPNA isoform would considerably increase specificity. Pioneering work in the search for Karyopherin alpha/beta 1 inhibitors was started by Lin *et al.* in 1995. They among others, found that a cell-permeable peptide containing the NLS of the NFκB p50 subunit was able to inhibit the nuclear import of transcription factors containing a NLS, such

as NFκB, NFAT, AP-1 and STAT1 [76, 200]. The peptide became better known as cSN50.1 and more recently has been found to target both SREBP/KPNB1 and KPNA/NLS-cargo mediated import through two separate mechanisms. The inhibition of SREBP/KPNB1-mediated import is thought to be through the binding of the peptide's SSHR motif with KPNB1. The peptide is also able to bind all KPNA isoforms, except KPNA6, with the highest affinity for KPNA1 and in that way plays a role in the import inhibition of transcription factors containing a NLS [77]. Kosugi *et al.* (2008) also used peptide inhibitor design to target the KPNA/B1 import pathway which yielded two high affinity peptides namely; Bimax1 and Bimax2. They were found to specifically target the KPNA-mediated import pathway and had no effect on KPNB1-only mediated import although they showed no specificity for Karyopherin alpha isoforms in mammalian cells [112]. The chemotherapeutic ability of these import inhibitors however, remain unknown at present. An anti-parasitic antibiotic, Ivermectin, already on the market, has also been found to bind non-specifically to KPNB1/KPNA preventing binding to the cargo proteins [193]. Although Ivermectin has been shown to have anticancer effects this seems to be through a mechanism of action unrelated to nuclear import inhibition [201].

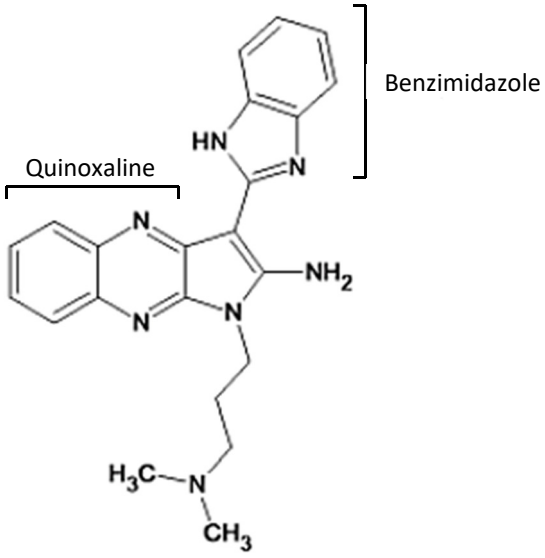
Karyostatin 1A was the first small molecule inhibitor of KPNB1-mediated nuclear import to be described. Its mechanism of action is thought to be through blocking the binding of RanGTP to KPNB1 disrupting the KPNA/B1 import pathway [194]. The potential of this compound including its anti-cancer effect has not been explored to date. Another small molecule inhibitor of nuclear import, Importazole, developed by Soderholm *et al*. (2011) was found to interfere with the interaction between RanGTP and KPNB1 and specifically disrupts KPNB1-mediated import without affecting Transportin or XPO1-mediated transport [195]. Importazole successfully inhibited NFκB p65 nuclear localisation in myeloma cells and induced apoptosis without affecting normal plasma cells, showing promise as an anti-cancer compound [202]. More recently INI-43, a compound described by our research group, was

shown to inhibit KPNB1-mediated nuclear import in cancer cells causing cell death and reducing tumour size *in vivo* [203] (Data from this study was included in the publication).

1.10 Identification of the nuclear import inhibitor: INI-43

A structure-based *in silico* drug screen was performed in the search of identifying potential novel small molecules that would bind to KPNB1 and inhibit nuclear import. This was done in collaboration with researchers at the James Graham Brown Cancer Centre (University of Louisville, Kentucky, USA). The overlapping region on KPNB1 where Karyopherin alpha and RanGTP bind, corresponding to amino acids 331-363, is thought to be essential for KPNB1 function and deletion of this region rendered KPNB1 unable to transport cargo into the nucleus [204]. Using the known crystal structure of KPNB1, a compound library consisting of 12 662 570 small molecules was screened for their predicted ability to bind this overlapping region on KPNB1. Seventy-four compounds were identified from the search and numbered according to their predicted binding affinity to KPNB1. A number of compounds were screened for their ability to kill cancer cells and block the nuclear import of cargo proteins and of these, Compound 43, *3-(1H-1,3-benzimidazol-2-yl)-1-[3-(dimethylamino)propyl]-1H-pyrrolol[2,3-b]quinoxalin-2-amine,* which was later renamed Inhibitor of Nuclear Import-43 (INI-43) was selected for further investigation. This compound showed the ability to inhibit nuclear import of KPNB1-cargoes and had half maximal response concentration (EC_{50}~ 10 μM) values in cervical cancer cell lines which were in the range of current chemotherapeutics e.g. cisplatin and doxorubicin when used on cancer cells in culture. The small molecule inhibitor was found to associate with changes in KPNB1 localisation and expression levels. INI-43 has a quinoxaline group, a heterocyclic molecule consisting of a benzene and a pyrazine ring, as well as a benzimidazole side chain, a bicyclic compound with a imidazole and benzene fused

together (Fig. 1.5). In this study we aim to further characterise the anticancer properties of INI-43 and its inhibitory effect on nuclear import [203].



Mono-isotopic mass: 385.2 g/mole

Figure 1.5: Structure of the small molecule Inhibitor of Nuclear Import -43 (INI-43). A quinoxaline derived compound containing a benzimidazole side chain with a mono-isotopic mass of 385.2 g/mole.

1.11 Significance

The nuclear import protein, KPNB1, has been shown to have potential as an anticancer target. Cancer cells have many biological phenotypes that differ from non-cancer cells. There is growing importance that inflammation promotes many aspects of cancer progression, hence mechanisms for inhibiting inflammatory signalling in the tumour microenvironment might be favourable. Anti-inflammatory drugs may have a role in preventing malignancies but are generally not cytocidal [42]. An ideal drug would be able to suppress inflammation and kill cancer cells simultaneously. Targeting KPNB1 has the potential to inhibit the nuclear entry and activity of cargoes associated with cancer biology.

1.12 Project aims

This study is aimed at investigating the role of the nuclear import protein, KPNB1, in cancer cell biology and the transport of transcription factors associated with inflammatory signalling in cancer cells.

The objectives for the investigation were:

i) To determine the effect of KPNB1 inhibition on cervical cancer cell biology including; proliferation, survival, migration and invasion.

ii) To determine whether KPNB1 is required for the nuclear import, activity and inflammatory signalling of the transcription factors; NFkB and AP-1.

iii) To investigate the anticancer role of the nuclear import inhibitor, INI-43, in an ectopic xenograft mouse model.

CHAPTER 2

INVESTIGATING THE EFFECTS OF NUCLEAR IMPORT INHIBITION VIA KARYOPHERIN BETA 1 ON CANCER CELL BIOLOGY

2.1 INTRODUCTION

The nucleo-cytoplasmic transport system plays an essential role in normal cell functioning and defects in this system have been associated with cancer. Dysregulation of proteins in the transport system can be attributed to various factors. These include; altered expression or localisation of the transport proteins, disruption of endogenous inhibitors and the mechanistic implications of nuclear transporters in mitotic division and genetic instability [119, 205]. Some of the key hallmarks in the development of cancer include sustained proliferation and the activation of invasion, therefore targeting these phenotypes are considered desirable when designing targeted chemotherapeutic drugs [34].

Our research group has focused on investigating the potential of KPNB1 as an anticancer target. This is based on studies showing that the expression of nuclear import proteins was upregulated in cervical cancer patient tissue in comparison to normal tissue [128]. The overexpression of KPNB1 in cervical cancer was found to be caused by altered transcriptional regulation by E2F. Elevated E2F activity in cervical cancer is attributed to the human papillomavirus E7 protein known to inhibit the retinoblastoma protein which would normally repress E2F activity [129]. Overexpression of the nuclear transport protein has been shown to

increase nuclear import efficiency in transformed cells [133]. This enhanced access of cargo into the nucleus is suggested to sustain the high metabolic and proliferative demands of cancer cells. Cancer cells have been found to require KPNB1 for their proliferation, however the exact mechanism of action for this requirement is not fully understood [128, 138, 203]. It could be attributed to either; the essential role of KPNB1 in mitosis and/or to the inability of certain cargoes e.g. transcription factors to gain access into the nucleus and perform their function in promoting proliferation [205]. Because of the central role that KPNB1 plays in the cell it is likely that its inhibition will affect multiple biological phenotypes of cancer cells. Included in the biological phenotypes of cancer cells are; increased proliferation and changes in cancer cell migration and invasion. While there is evidence that KPNB1 is necessary for the proliferation and survival of cancer cells [128], little is known about the role of KPNB1 in the progression of other cancer phenotypes such as migration and invasion.

Once the proliferative demands of cancer cells outweigh their nutrient supply they acquire invasive properties to escape the primary site of the tumour and occupy secondary locations. This is achieved through the expression of matrix-modifying enzymes to degrade the extracellular matrix thereby facilitating migration and invasion [34, 206]. Matrix metalloproteases -2 and -9 (MMP-2 & -9) have both been identified as key activators of cancer cell invasion [207, 208]. Dysregulation of the MMPs and their respective endogenous inhibitors; TIMP-2 and TIMP-1, might also contribute to cancer progression [104, 207]. In this study, we used transwell migration and invasion assays to analyse changes in motility following KPNB1 inhibition. The mechanism behind such changes was further investigated by measuring MMP and TIMP expression as well as assessing MMP activity following nuclear import inhibition.

This chapter aims to further investigate the role of KPNB1 in cancer cell biology using cervical cancer cell lines as a model system. This includes investigating the effects of KPNB1 on biological endpoints including; cancer cell proliferation, survival, migration and invasion. This study uses two approaches to inhibit KPNB1, i) KPNB1 siRNA to directly target KPNB1 and ii) treatment with the small molecule Inhibitor of Nuclear Import, INI-43, previously reported to inhibit nuclear import associated with KPNB1 [203]. In doing so we aim to further characterise INI-43 as a potential anticancer drug. Our data suggests that not only does KPNB1 play a role in cancer cell proliferation and survival but also in migration and invasion.

2.2 RESULTS

2.2.1 The role of KPNB1 in cancer cell proliferation and survival

To monitor the effects of KPNB1 inhibition on cervical cancer cells, HeLa cell proliferation was analysed following nuclear import inhibition using KPNB1 specific siRNA as well as the small molecule inhibitor; INI-43.

2.2.1.1 The effect of KPNB1 siRNA knockdown on cancer cell proliferation

In order to determine whether KPNB1 was essential for cancer cell proliferation a pool of interfering RNA's specific for KPNB1 was used to inhibit KPNB1 activity. HeLa cervical cancer cells were transfected with control and KPNB1 siRNA and allowed to proliferate over several days. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was used to measure cell proliferation over 24, 48 and 72 hours. Our results show a significant reduction in cell proliferation within 48 hours post-transfection with KPNB1 siRNA onwards indicating reliance on KPNB1 for cancer cell proliferation (Fig. 2.1 A). Knockdown of KPNB1, 48 hrs post transfection, was checked in a parallel experiment by western blotting (Fig. 2.1 B).

Having shown that KPNB1 knockdown with siRNA inhibited HeLa cervical cancer cell proliferation, we next investigated the effects of the small molecule, INI-43, on cervical cancer cells.

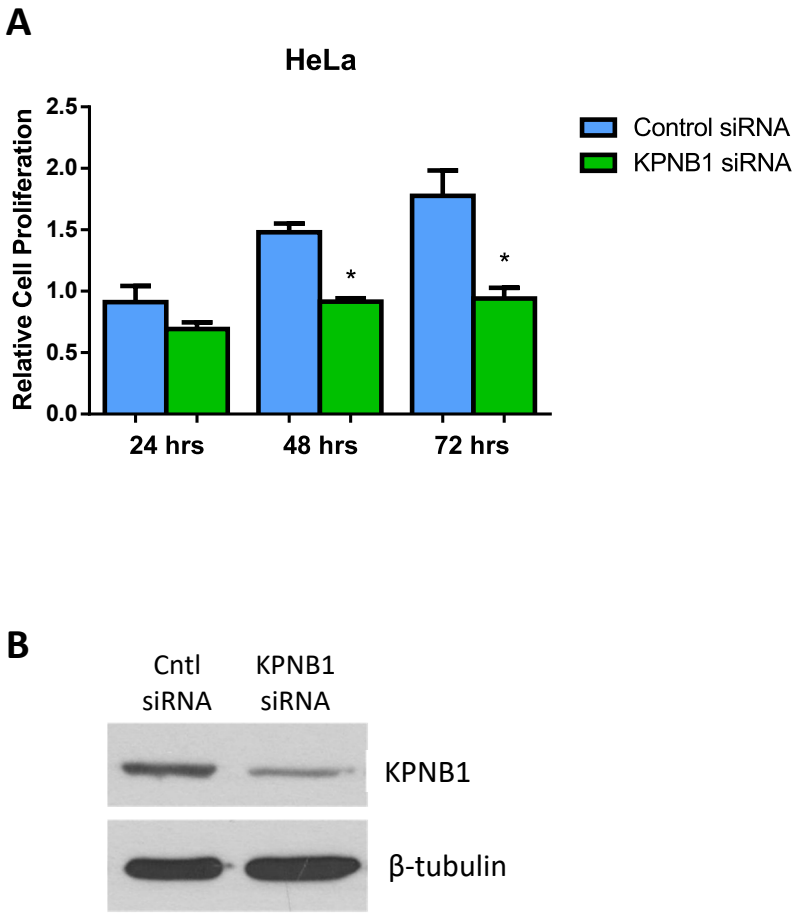


Figure 2.1: The effect of KPNB1 knockdown by KPNB1 siRNA on cancer cell proliferation. A) Hela, cervical cancer cells, were transfected with control and KPNB1 siRNA and cell proliferation measured using an MTT proliferation assay at 24, 48 and 72 hour timepoints. **B)** Western blot showing KPNB1 knockdown 48 hrs post transfection. Results shown are the mean ± SEM of experiments performed in triplicate and repeated at least two independent times. (*p<0.05).

2.2.1.2 EC_{50} determination for INI-43 in cervical cancer cells and normal fibroblasts

As we have evidence suggesting that INI-43 targets KPNB1 [203], it was important to determine the effective concentration at which INI-43 kills 50% of viable cells for use in subsequent experiments aimed at determining its effects on cancer cell biology. A panel of cervical cancer cell lines including; HeLa, SiHa and CaSki, were used as well as non-cancer FGo fibroblast cells. Cells were treated with a range of nine INI-43 concentrations from 1 µM to 50 µM. The OD_{595nm} absorbencies obtained from the MTT cell viability assay were plotted as sigmoidal dose-response curves (Hill Plots) using GraphPad Prism and the EC_{50} calculated (Fig 2.2). The representative EC_{50} values for all cell cultures including their confidence intervals were tabulated (Table 2.1). The cervical cancer cell lines had a very similar EC_{50} value for INI-43 with HeLa at 7.1 µM, SiHa at 9.8 µM and CaSki at 7.3 µM. For all future experiments 5 or 10 µM INI-43 was chosen to treat cervical cancer cells. The non-cancer, FGo cells, appeared to be slightly less sensitive to INI-43 with an EC_{50} value of 17.0 µM, approximately double that of the cancer cells (Table 2.1).

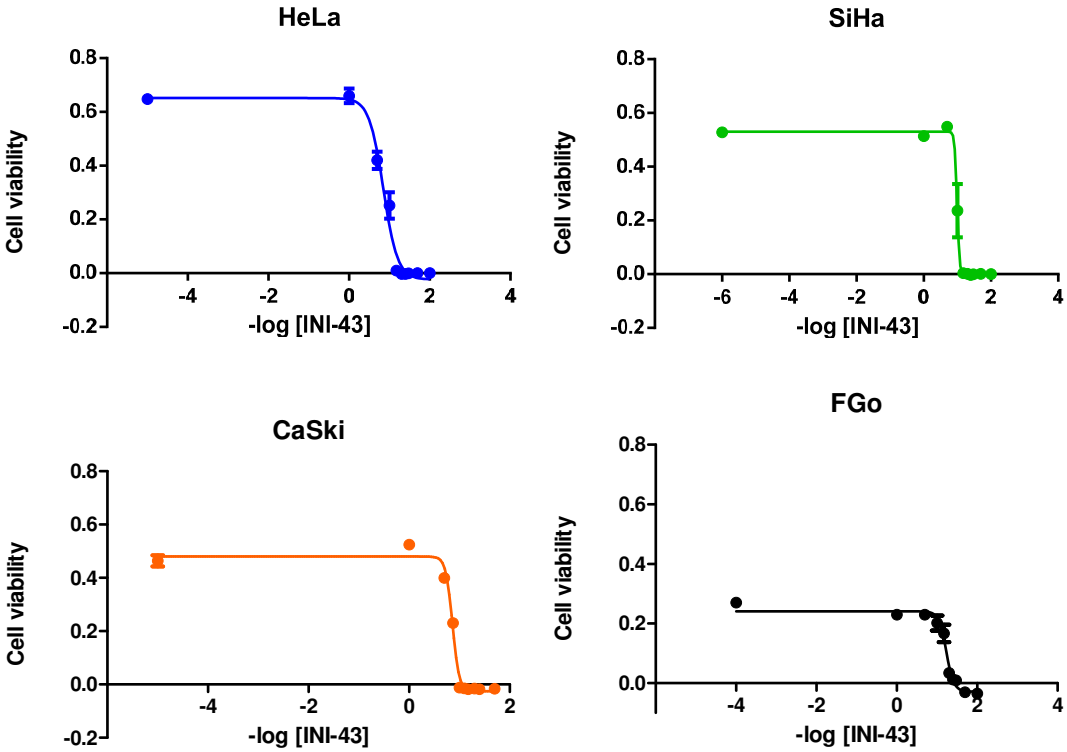


Figure 2.2: EC_{50} determination for INI-43 in cervical cancer cell lines and non-cancer cells. Cervical cancer cells (HeLa, SiHa and CaSki) and non-cancer fibroblast cells (FGo) were seeded into 96-well plates in triplicate. Cells were treated with a range of INI-43 concentrations and MTT added after 48 hours and the absorbance read at OD_{595nm}. Transformed -log[INI-43] graphs were used to determine the EC_{50} concentration of INI-43 in each of the cell cultures. Results shown are the mean ± SEM of experiments performed in triplicate performed three independent times. (*p<0.05).

TABLE 2.1: Mean EC_{50} values for INI-43

Cells	EC_{50} Value	Confidence Interval
HeLa	7.1 µM	6.2 -8.2 µM
SiHa	9.8 µM	8.9 -10.9 µM
CaSki	7.3 µM	6.9 -7.6 µM
FG_0	17.0 µM	15.5 -18.7 µM

2.2.1.3 KPNB1 inhibition induces cancer cell death via apoptosis

KPNB1 knockdown or INI-43 treatment of cervical cancer cells resulted in significant amounts of cell death at the concentrations used. In order to investigate whether the mechanism of cell death was occurring via apoptosis, a Caspase-Glo® assay was performed looking at caspase 3/7 activity. The cysteine aspartic acid-specific proteases -3 and -7 (caspase-3 and -7) play an effector role in the activation of apoptosis in mammalian cells [209, 210]. The Caspase-Glo® assay provides a lumogenic, tetrapeptide (DEVD) substrate for caspase 3/7. Cleavage of the substrate by the caspases releases aminoluciferin, a luciferase substrate, thereby producing a measurable light signal. The intensity of the light signal is proportional to caspase 3/7 activity. HeLa cells were transfected with control and KPNB1 siRNA and caspase 3/7 activity measured after 24, 48 and 72 hours. A significant increase in caspase 3/7 was observed across all time points measured after transfection with KPNB1 siRNA (Fig. 2.3 A).

To monitor the effect of the small molecule inhibitor of nuclear import, HeLa cells were treated with 10 µM INI-43 and caspase 3/7 activity analysed after 3, 6 and 24 hours of treatment. Significant caspase 3/7 activity was observed at 6 hours and 24 hours post INI-43 treatment (Fig. 2.3 B). A 3 hour timepoint, before significant cell death occurred, was therefore used in future functional experiments looking at the effects of nuclear import inhibition. These results show that inhibiting KPNB1 using both siRNA and INI-43 induces cell death via apoptosis.

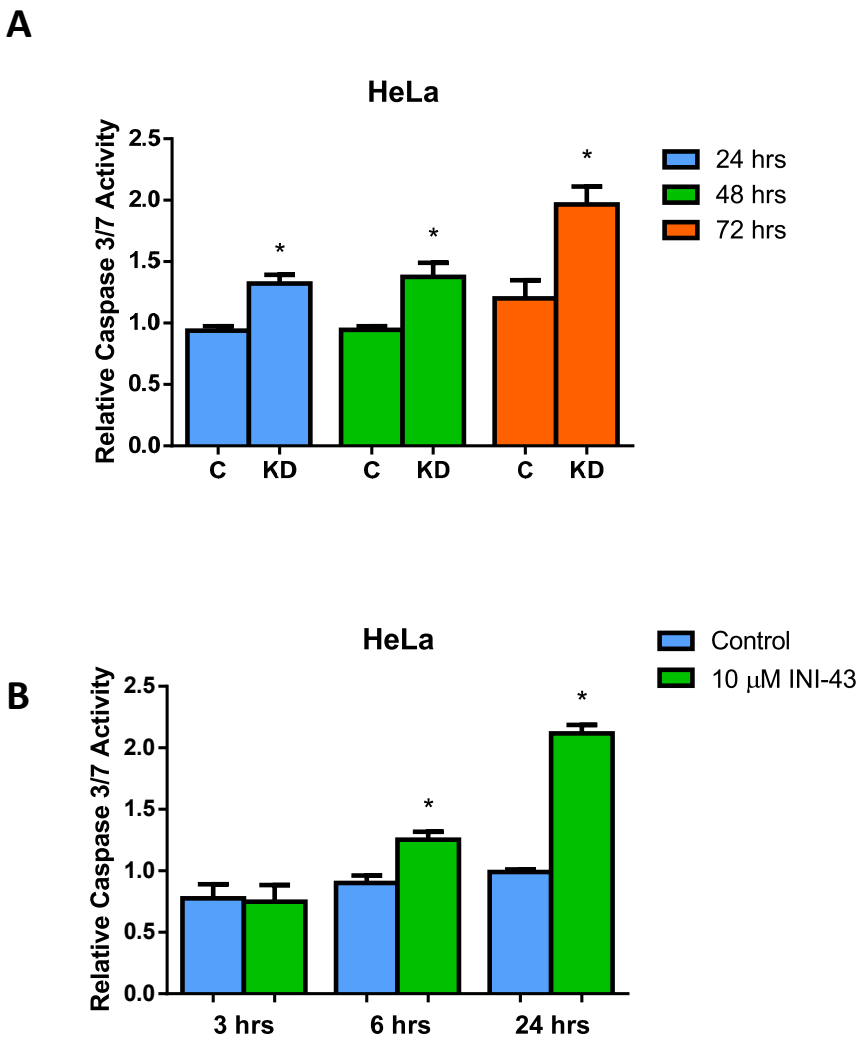


Figure 2.3: KPNB1 inhibition induces cell death by apoptosis. A) HeLa cells were transfected with control and KPNB1 siRNA and Caspase 3/7 activity measured over 72 hours using the Caspase 3/7 GLO® assay. C= control siRNA, KD= KPNB1 knockdown. Results were normalized to a concurrent MTT cell viability assay. **B)** HeLa cells were treated with 10 µM INI-43 and Caspase 3/7 activity measured over 24 hours. Results were normalized to a concurrent MTT cell viability assay. Results shown are the mean ± SEM of experiments performed in triplicate and repeated two independent times. (*p<0.05).

2.2.1.4 The effect of INI-43 on PMA-stimulated cancer cell proliferation

The phorbol ester, PMA, is a known stimulator of the cancer phenotype by activating various signalling pathways associated with proliferation, migration, invasion and protection against apoptosis [211-213]. In an *in vitro* cell culture system PMA can be used to mimic such intra-tumour signalling in order to determine whether this effect can be reversed. To investigate whether INI-43 could inhibit PMA-stimulated cancer cell proliferation, the cell viability of HeLa cells was measured over a range of INI-43 concentrations in the presence of PMA over 24 hours. The results show that PMA treatment caused a small but significant increase in cancer cell proliferation but not in the presence of INI-43 at concentrations higher than 5 µM (Fig. 2.4 A).

As subsequent functional experiments assessed whether inhibition of KPNB1 inhibited PMA-stimulated cancer phenotypes it was interesting to determine whether the EC_{50} value of INI-43 might change in the presence of PMA. HeLa cells were again treated with a range of INI-43 concentrations from 0 µM to 50 µM over 24 hrs in the presence or absence of 100 nm PMA. A sigmoidal dose-response curve was plotted to calculate the effective concentration at which 50% of the cells were viable. The EC_{50} value for INI-43 shifted slightly from 5.4 µM to 6.0 µM in the presence of PMA and although the confidence intervals do not overlap a change this small in a clinical setting would most likely be deemed insignificant (Fig. 2.4 B).

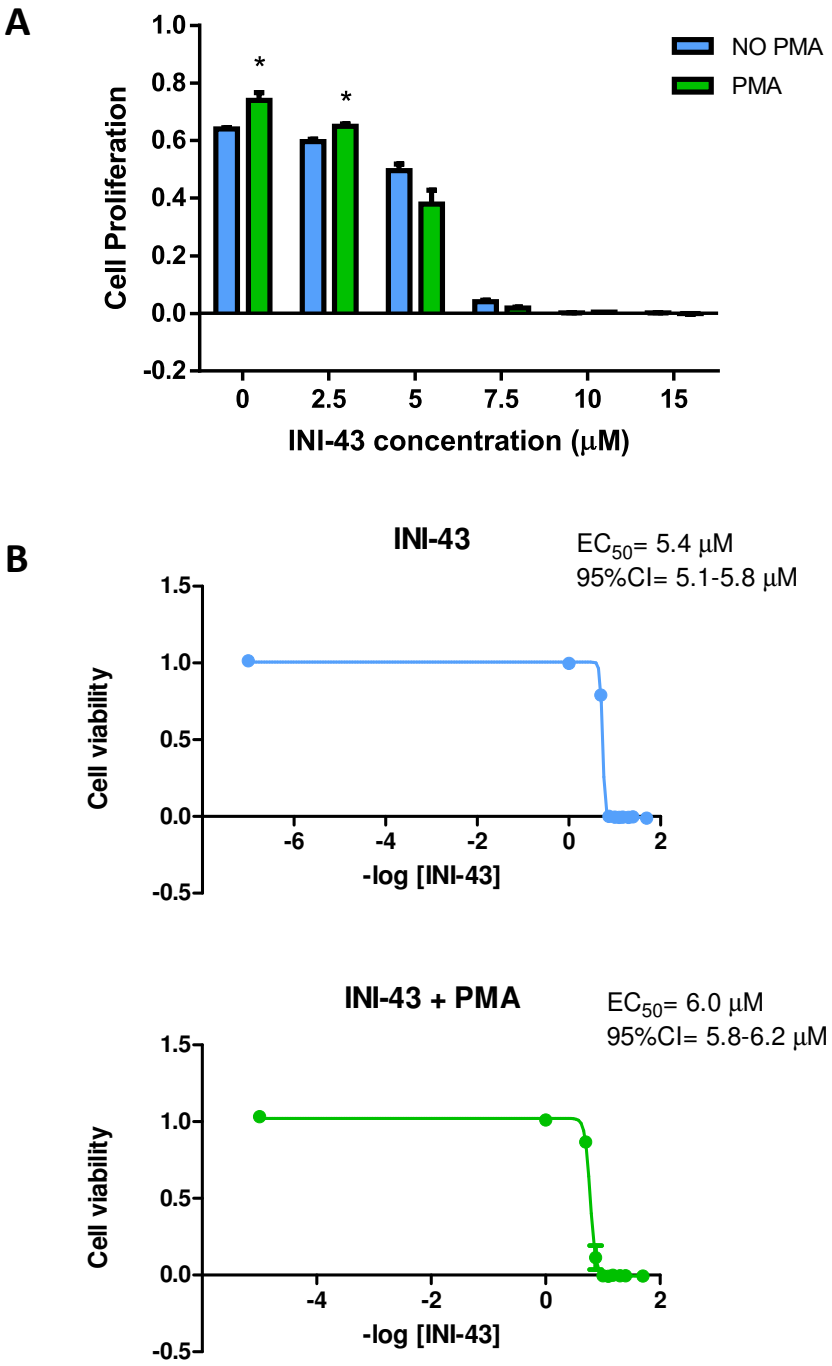


Figure 2.4: The effect of INI-43 on PMA-stimulated cell proliferation. A) HeLa cells were treated with a range of INI-43 in the presence and absence of 100 nM PMA over 24 hours. Cell proliferation was measured using the MTT cell viability assay. **B)** The EC_{50} of INI-43 in HeLa cells was measured after 48 hours in the presence and absence of 100 nM PMA and the 95% confidence interval shown. Results shown are the mean ± SEM of experiments performed in triplicate and repeated two independent times. (*p<0.05).

2.2.2 The role of KPNB1 in cancer cell migration and invasion

While the effect of KPNB1 inhibition on cancer cell proliferation and survival is well characterised (Section 2.2.1) [128, 203], little is known about its role in other cancer phenotypes such as migration and invasion. In this study, we investigated the requirement of KPNB1 expression and activity on cancer cell migration and invasion using transwell motility assays. The expression and activity of key matrix-modifying enzymes that have been reported to associate with migration and invasion; MMP-2 and MMP-9, as well as endogenous inhibitors; TIMP-2 and TIMP-1 were also assessed following KPNB1 inhibition.

2.2.2.1 The effect of KPNB1 inhibition on cancer cell migration

To determine the migratory ability of cancer cells a transwell migration assay was used. This assay allows cells with a migratory potential to move from the upper chamber in which they are placed, through the 8 µm pores towards a chemoattractant, high serum concentration, in the lower chamber. Migrated cells were then stained, photographed and quantified. Hela cells transfected with control or KPNB1 siRNA were placed in the upper chamber in the presence of PMA and allowed to migrate through the membrane overnight. Results are representative images of cell migration assays and shows fewer cells migrating following KPNB1 knockdown (Fig. 2.5 A). Knockdown of KPNB1 was confirmed by western blotting (Fig. 2.5 B). As KPNB1 inhibition is known to cause cell death, a concurrent MTT viability assay at the same time point was performed to confirm that the results observed were due to effects on cell migration rather than due to cell death through KPNB1 inhibition and migrated cells corrected for live cells. The number of cells migrating was quantified and expressed relative to control. Quantification of the data shows that KPNB1 knockdown significantly inhibited the migration of HeLa cells (Fig. 2.5 C).

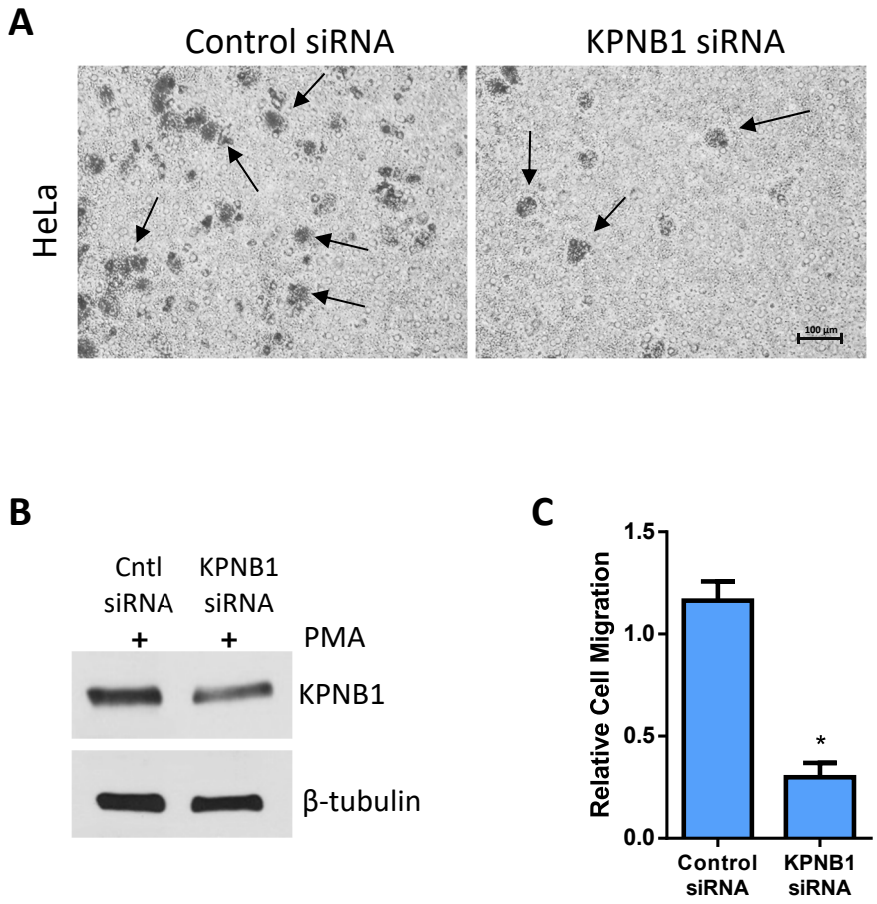


Figure 2.5: The effect of KPNB1 knockdown on the migratory potential of cancer cells. A) Representative images from the transwell migration chamber showing migration of HeLa cells following KPNB1 knockdown, arrows identifying migrating cells. Scale bar= 100µm, magnification x100. **B)** Western blot confirming KPNB1 knockdown. **C)** Quantification of transwell migration assay following KPNB1 knockdown normalised to MTT cell viability. Results shown are the mean ± SEM of experiments performed in triplicate and repeated two independent times. (*p<0.05).

Next, we investigated whether INI-43 had a similar effect to KPNB1 knockdown on cancer cell migration in a panel of cervical cancer cell lines. Representative images taken of the transwell membranes shows fewer Hela, SiHa and CaSki cells migrating following INI-43 treatment (Fig. 2.6 A). A treatment time of 3 hours was selected as INI-43 had no effect on HeLa cell proliferation at this timepoint (Fig. 2.3 B). Although a treatment duration of INI-43 was kept to 3 hours, before cell death was evident, concurrent MTT cell viability assays were performed alongside the migration assay to correct for any possible effect that may have been due to cell death. Overall migrated cells were quantified which showed that INI-43 significantly reduced

the migratory ability of HeLa and SiHa cells (Fig. 2.6 B). While not significant in CaSki cells, a trend towards reduced migratory ability following INI-43 treatment was observed.

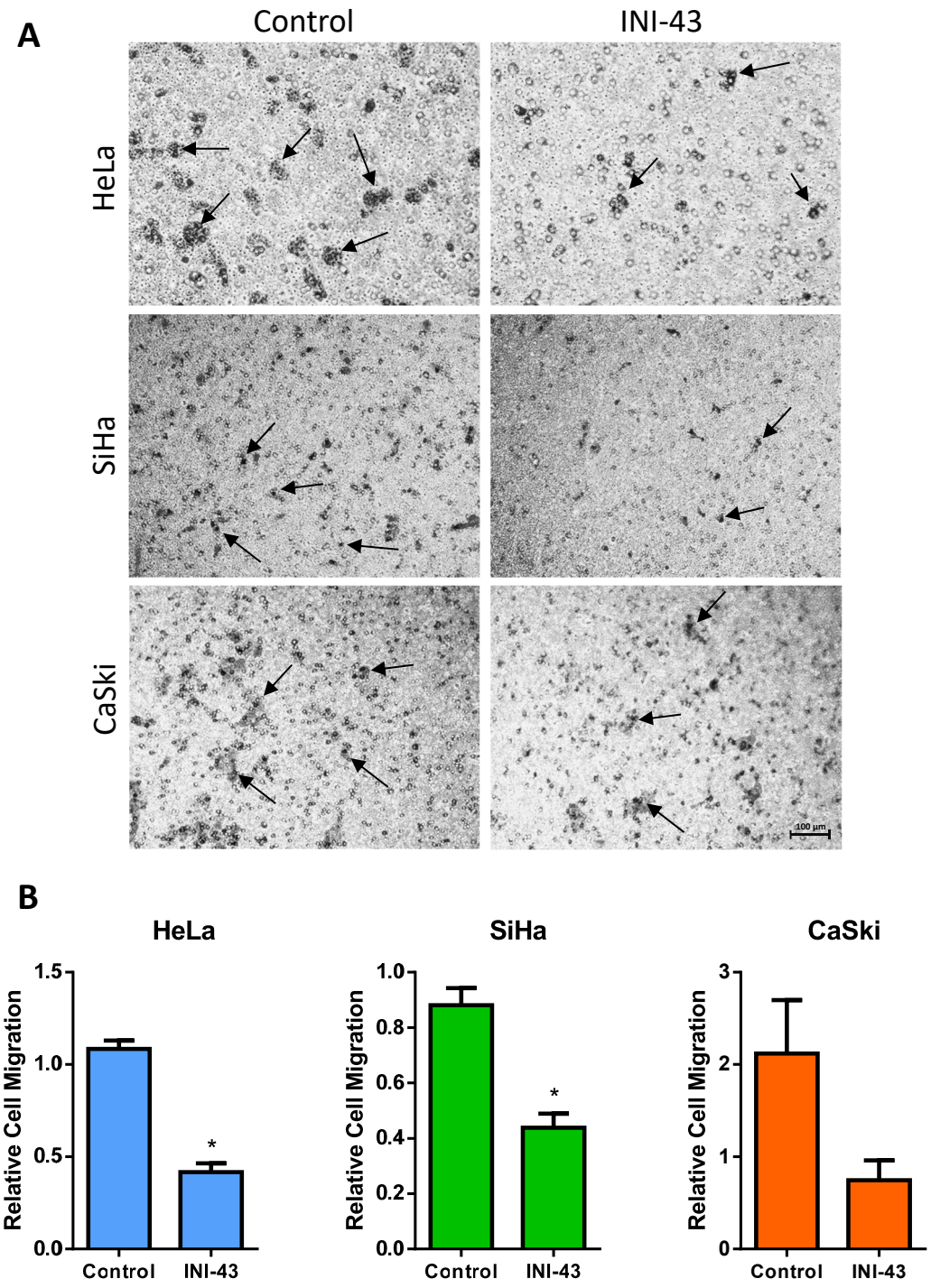


Figure 2.6: The effect of INI-43 on the migratory potential of cancer cells. A) Representative images showing HeLa, SiHa and CaSki cell migration through the membrane following a 3 hour 10 µM INI-43 pre-treatment, arrows identifying migrating cells. Scale bar= 100µm, magnification x100. **B)** The relative number of HeLa, SiHa and CaSki cells that migrated through the transwell chamber following INI-43 treatment were quantified and normalised to MTT cell viability. Results shown are the mean ± SEM of experiments performed in triplicate and repeated two independent times. (*p<0.05).

2.2.2.2 The effect of KPNB1 inhibition on cancer cell invasion

Having observed that KPNB1 was required for migration of cancer cells we next questioned whether it was required for cancer cell invasion. A transwell assay similar to the migration assay described above, but with the addition of a matrigel coating on the transwell membrane, was used. Representative images show fewer cells were able to invade through the matrigel following KPNB1 inhibition using siRNA (Fig. 2.7 A & B). The number of invaded cells was quantified and results show a significant reduction in cell invasion following KPNB1 inhibition (Fig. 2.7 B).

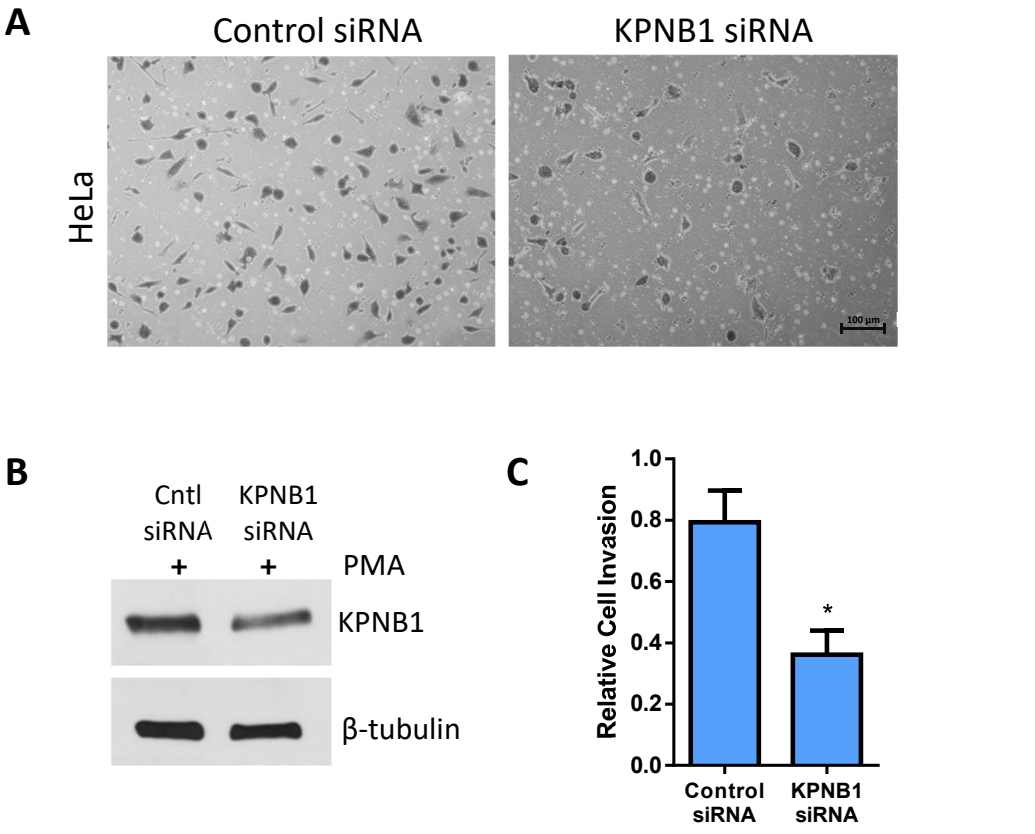


Figure 2.7: Effect of KPNB1 knockdown on cervical cancer cell invasion. A) Representative images of the invasion transwell assay showing invasion of HeLa cells through the matrigel-coated transwell membrane following KPNB1 knockdown. Scale bar= 100µm, magnification x100. **B)** Western blot confirming KPNB1 knockdown. **C)** Quantification of the invasion assay following KPNB1 knockdown normalised to MTT cell viability. Results shown are the mean ± SEM of experiments performed in triplicate. (*p<0.05).

Cervical cancer cells treated with INI-43 to inhibit nuclear import showed similar results as KPNB1 knockdown experiments. Representative images in Figure 2.8 (A), shows considerably fewer HeLa and SiHa cells invaded the matrigel-coated transwell chamber following INI-43 treatment. Overall cell counts of invading cells were used to quantify the inhibitory effects of INI-43 on invasion. INI-43 was able to significantly inhibit cancer cell invasion of both HeLa and SiHa cells (Fig. 2.8 B). Together these results provide evidence that the inhibition of KPNB1 by both siRNA and INI-43 treatment inhibits multiple biological phenotypes of cervical cancer cells including; proliferation, migration and invasion.

2.2.2.5 The effect of INI-43 on the expression of gelatinases and their endogenous inhibitors in cancer cells

The ability of cells to invade surrounding tissues depends on the expression and activity of matrix metalloproteases (MMP's). These enzymes degrade the extracellular matrix (ECM) and pave the way for cell migration and invasion. Tissue inhibitors of matrix metalloproteases (TIMPs) effectively inhibit MMP activity and it can therefore be hypothesized that the expression of MMP's and TIMP's be inversely regulated. qRT-PCR was used to assess the expression of MMP-2 and -9 as well as TIMP-1 and -2 following INI-43 treatment. Interestingly, INI-43 treatment resulted in a significant reduction in the mRNA expression of MMP-2 and MMP-9 (Fig. 2.9 A & B) while the expression of TIMP-1 and TIMP-2 was significantly up-regulated (Fig. 2.9 C & D). In our experiments, PMA was unable to significantly enhance MMP or repress TIMP expression as suggested in the literature possibly due to the short treatment duration of only 3 hours in HeLa cells used in our experimental conditions. This data supports the inhibitory effect of nuclear import inhibition on cancer cell invasion previously seen using the transwell assays.

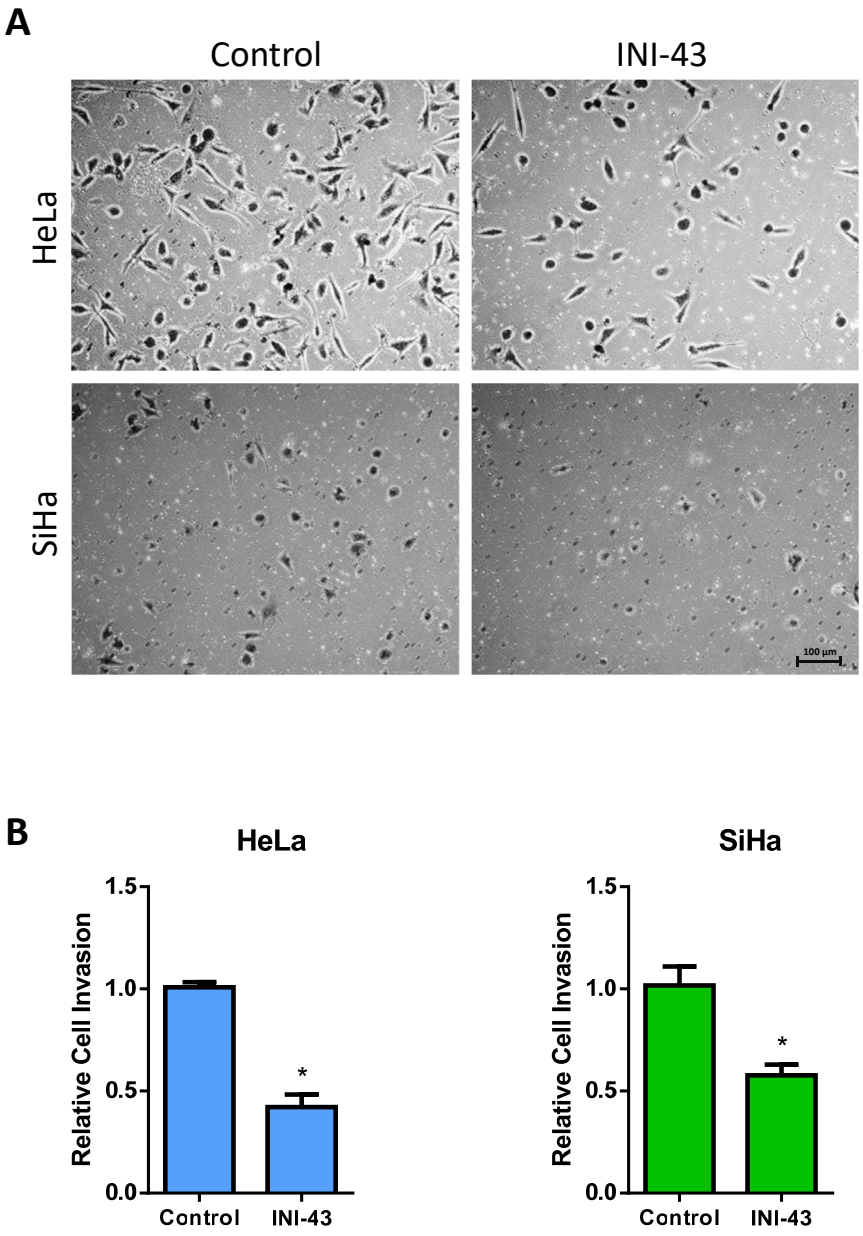


Figure 2.8: Effect of INI-43 on cervical cancer cell invasion. A) Representative images of the invasion assay following 3 hr, 10 µM INI-43 pre-treatment of HeLa and SiHa cells showing the number of cells able to invade the matrigel-coated membrane following nuclear import inhibition. Scale bar= 100µm, magnification x100. **B)** Quantification of invasion assay following INI-43 treatment, normalised to MTT cell viability. Results shown are the mean ± SEM of experiments performed in triplicate. (*p<0.05).

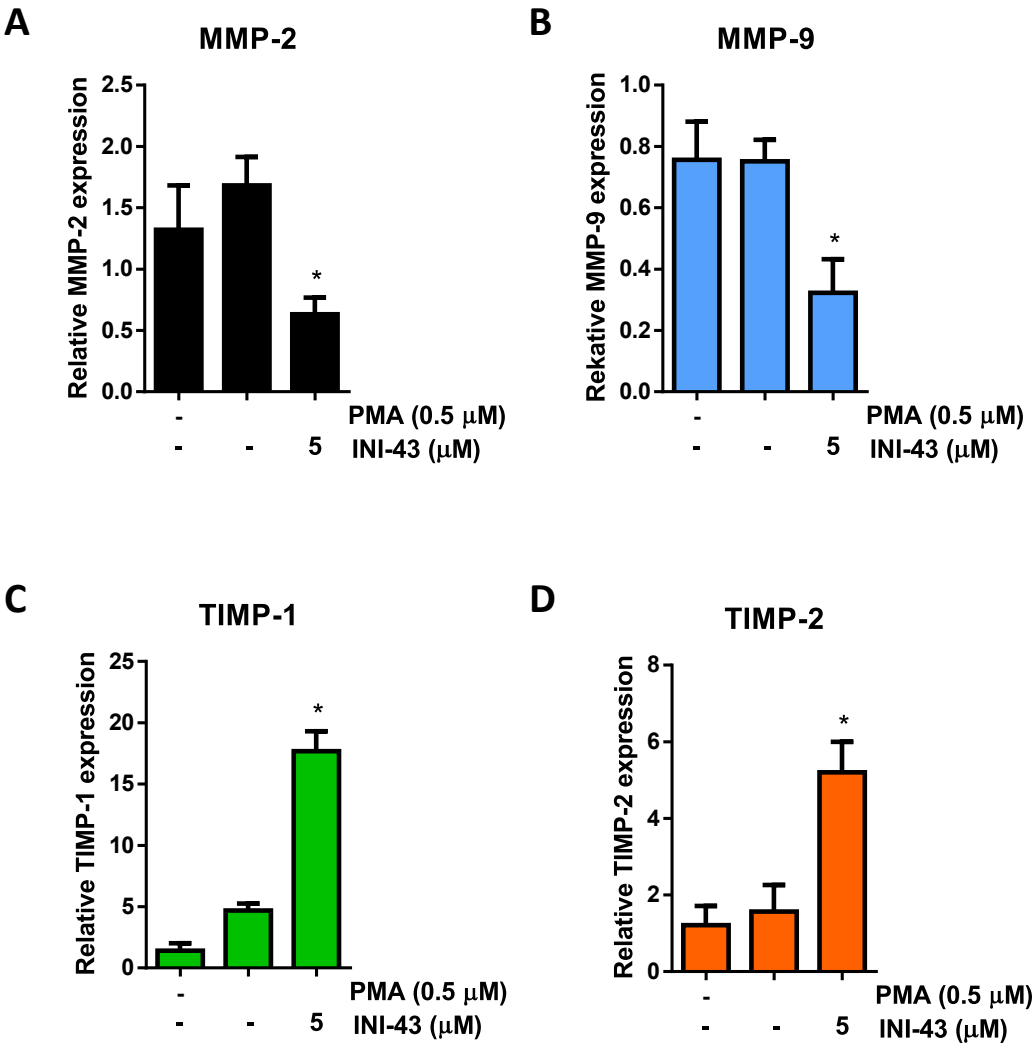


Figure 2.9: Effect of INI-43 on MMP-2, MMP-9, TIMP-1 and TIMP-2 expression. qRT-PCR analysis of mRNA expression following 5 µM INI-43 treatment for 21 hours with the addition of 0.5 µM PMA for a further 3 hours in HeLa cells shown for MMP-2 **(A)**, MMP-9 **(B)** and inhibitors of matrix metalloproteases, TIMP-1 **(C)** and TIMP-2 **(D)**. Results shown are the mean ± SEM of experiments performed in triplicate and repeated three independent times. (*p<0.05).

2.2.2.6 The effect of INI-43 on MMP-9 gelatinase activity in cervical cancer cells

Decreased levels of MMP mRNA expression suggests but doesn't confirm that MMP activity is also affected by INI-43 treatment. A Gelatin Zymography was used to determine whether nuclear import inhibition through INI-43 affected the activity of MMP-9. To measure MMP activity, conditioned media from cervical cancer cells was collected and used in the Gelatin Zymography. Our results showed a slight increase in MMP activity following PMA treatment in HeLa and SiHa cells (Fig. 2.10 A). CaSki cells had lower MMP activity in comparison. Treatment with INI-43, had a substantive inhibitory effect on MMP activity in all three cervical cancer cell lines; HeLa, CaSki and SiHa. Quantification of the zymograms by densitometry for MMP-9 activity following INI-43 treatment is shown in Fig. 2.10 (B). These results support that treatment with INI-43 inhibits MMP-9 expression and activity.

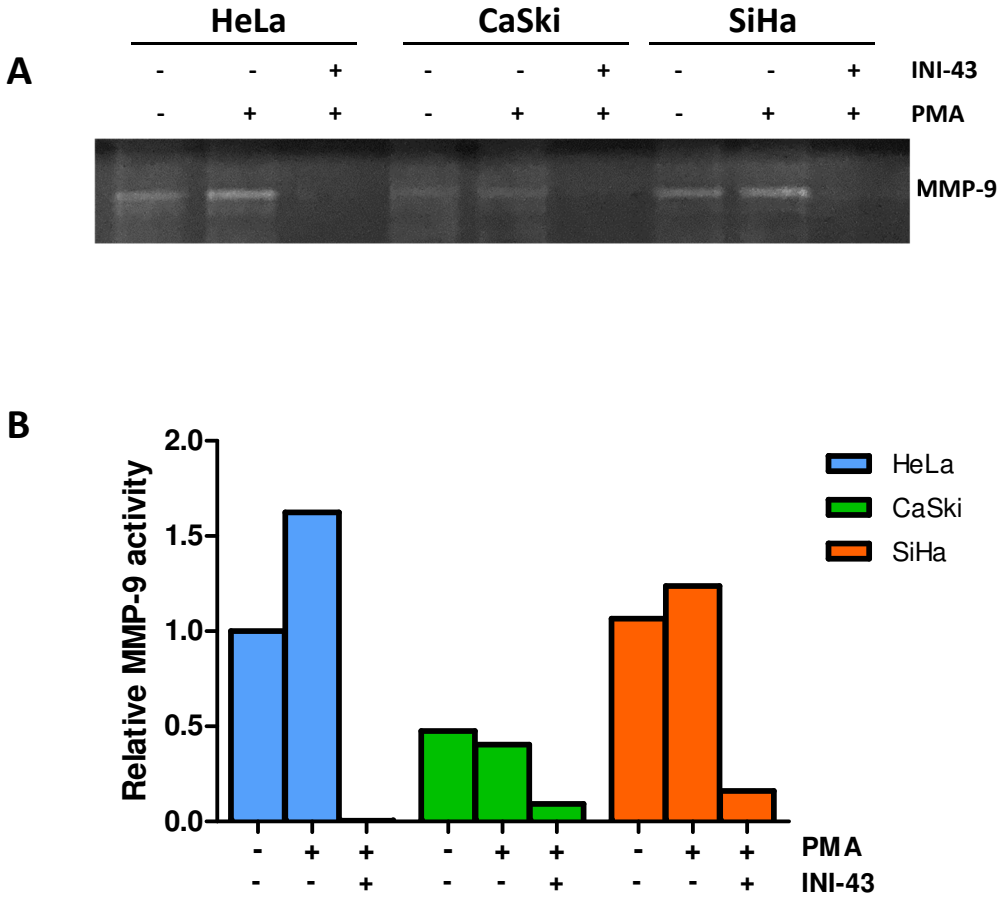


Figure 2.10: Effect of INI-43 on gelatinase activity of MMP-9 in cervical cancer cells (A) Gelatin Zymography showing MMP-9 activity in HeLa, CaSki and SiHa conditioned media collected after 16 hours following a 3 hour pre-treatment of 10 µM INI-43 and a 1 hour 0.5 µM PMA stimulation. Regions of clearing indicate areas of gelatinase activity. **(B)** Quantification by densitometry of MMP-9 activity in three cervical cancer cell lines following INI-43 treatment and PMA stimulation. Results shown were repeated two independent times.

2.3 DISCUSSION

In recent years the nucleo-cytoplasmic transport system has gained interest as a targeted therapy for cancer. Drugs targeting the nuclear export protein, XPO1, have been successful in the treatment of various haematological cancers as well as solid tumours in animal models and in clinical trials [189, 214-219]. The Selective Inhibitor of Nuclear Export, Selinexor (KPT-330), covalently inhibits XPO1 and after showing promising preclinical anti-tumour activity is currently being used in several phase I, II and III clinical trials [191, 192]. There is increasing interest in the field of targeting nuclear import inhibitors specifically focusing on KPNB1-mediated transport. Supporting previous findings in our laboratory we found that KPNB1 is essential for cervical cancer cell proliferation. Non-cancer fibroblast cells, FGo, appear less sensitive to KPNB1 inhibition as a higher concentration of INI-43 was needed to achieve a level of cell death similar to that in cervical cancer cells. Van der Watt *et al.* (2009 & 2016) showed that normal fibroblasts, WI38, were less sensitive to KPNB1 knockdown than their transformed counterparts, SVWI38. Other non-cancer cells; CCD-1068SK, FGo and EPC2 epithelial cells, were reported to be unaffected by KPNB1 inhibition using siRNA [128, 203]. The greater dependency of cancer cells on KPNB1 compared to normal cells provides promise for KPNB1 as an anticancer target.

In this study, we report that inhibition of KPNB1 using both siRNA and INI-43 results in cancer cell death by apoptosis shown by an increase in Caspase 3/7 activity. The mechanism of cell death following KPNB1 inhibition using siRNA can be attributed to the activation of apoptosis via the intrinsic mitochondrial pathway as described by Angus *et al.* (2014). KPNB1 inhibition was found to induce prolonged M phase arrest accompanied by elevated Noxa expression and enhanced degradation of Mcl-1. This imbalance favours the pro-apoptotic response causing the translocation of Bax into the mitochondria, permeabilising the outer mitochondrial

membrane, releasing cytochrome c and activating various downstream effector caspases leading to apoptosis [186]. INI-43 treatment was similarly able to activate caspase 3/7 activity and induce apoptosis in cervical cancer cells following just 6 hours of treatment. A genome-wide loss-of-function study performed by Maartens-de Kemp *et al.* (2013) identified a cluster of genes that regulate the G_2-M phase transition and are essential to cell survival. Of these genes KPNB1 was found to be essential for tumour cell survival in head and neck and lung cancer cell lines [187].

Self-sufficiency from growth signals is a hallmark of cancer and accounts for cancer cell's unlimited proliferative potential within the tumour microenvironment. Cancer cells are thought to be able to produce their own growth signals which causes autocrine signalling stimulating proliferation [33]. The phorbol ester, PMA, can activate various signalling pathways that enhance proliferation [105, 106, 212]. We show that PMA increases the proliferation of cervical cancer cells and that INI-43 treatment inhibits PMA-stimulated proliferation at similar concentrations to when PMA was absent.

In our study we were interested in identifying the role of KPNB1 in cancer cell migration and invasion. We showed that inhibiting KPNB1 reduced the migration and invasion of cancer cells suggesting a requirement of KPNB1 in these biological phenotypes. As cancer cells require the activity of matrix-modifying enzymes to invade surrounding tissue, we investigated whether MMP activity was affected by KPNB1 inhibition. MMP activity was found to be significantly inhibited in cervical cancer cells following INI-43 treatment. Fan *et al.* (2016) found MMP-9 to be upregulated in cervical cancer tissue which correlated with a poor patient prognosis. Inhibiting MMP-9 activity in SiHa and HeLa cervical cancer cells reduced their migratory and invasive ability [220]. Similarly, MMP-2 expression was also found to be decreased following KPNB1 inhibition while expression of the inhibitors of matrix metalloproteases, TIMP-1 and

TIMP-2, were significantly increased. The role of TIMP 1 and 2 in cancer is less well defined and has been reported to play a differential role in different cancers [221]. TIMPs are known to be downregulated in a variety of cancer cell lines which is thought to contribute to the invasive properties of cancer cells. The overexpression of TIMP-2 in a highly metastatic melanoma cell line was able to inhibit metastasis [222, 223]. The matrix metalloproteases, MMP-2 and -9, have not only been associated with tumour invasion in breast cancer but also showed prognostic value where increased expression associated with poor patient survival [224, 225]. The inverse regulation of the MMPs and TIMPs following nuclear import inhibition by INI-43 as shown in our study suggests a switch in transcriptional regulation. Our results provide evidence that INI-43 has a suppressive effect on cancer cells in part by altering transcriptional patterns of genes associated with cell growth, migration and invasion. Together our data suggests that KPNB1 may play an important role in the migratory and invasive potential of cervical cancer cells. KPNB1 could be functioning as one of the key nuclear transporters of transcription factors associated with cancer cell migration, invasion and metastasis.

Given the potential for off-target effects of both siRNA and INI-43, these results could be validated using future experimental approaches including; RNAi reagents specific for KPNB1 or CRISPR/Cas mediated depletion of KPNB1.

CHAPTER 3

KARYOPHERIN BETA 1 IS REQUIRED FOR NFkB AND AP-1 ACTIVITY IN CERVICAL CANCER CELLS

3.1 INTRODUCTION

KPNB1 has multiple cellular roles with one of its main functions being the nuclear transport of transcription factors. Cell behaviour is driven by gene expression and gene expression is controlled by transcription factor activity. Transcription factors that have been associated with inflammation and cancer cell biology include the NFkB, AP-1 and STAT families of transcription factors. We hypothesised that the changes in cancer cell biology we observed when inhibiting KPNB1 may in part be a result of interfering with the nuclear import and hence activity of these key transcription factors required by cancer cells. We therefore investigated the effects of inhibiting nuclear import via KPNB1 on the nuclear entry and activity of NFkB and AP-1, as both have been implicated in acquiring the hallmarks of cancer.

NFkB is comprised of two subunits; p65 and p50, which both contain a nuclear localisation sequence (NLS), suggesting that they require a nuclear transport protein for their nuclear import [84]. There is contradicting evidence in the literature regarding the exact nuclear transporter; KPNA3/4, KPNB1, XPO7 or IPO8, for which NFkB is a cargo protein [83, 84]. We were thus interested in investigating whether KPNB1 is necessary for NFkB nuclear translocation and ultimately its transcriptional activity in cervical cancer cells. To monitor NFkB cellular localisation; Immunofluorescence, nuclear/cytoplasmic separation and electromobility shift assays were used. NFkB is responsive to PMA, which under normal

conditions induces nuclear localisation and therefore activation. Activity of the transcription factor was measured using transcriptional reporter assays and by assessing inflammatory target gene expression. As for NFkB, the transcription factor AP-1 has been reported to have a NLS present on the c-JUN subunit, although nuclear import of c-JUN is not limited to KPNB1 alone [226]. Nuclear transport proteins; Transportin 2 and Importin's 5, 7 and 9 are also reported to transport c-JUN [97]. AP-1 most commonly consists of c-JUN and c-FOS homo- and heterodimers and is activated through inflammatory cytokine signalling but also through PMA stimulation [88, 90]. Like for NFkB, several AP-1 target genes are implicated in the development and progression of cancer. We were therefore interested in determining whether KPNB1 may influence activity and target gene expression of the AP-1 transcription factor.

In this chapter, we investigated the effects of inhibiting KPNB1 and nuclear import using siRNA and the small molecule inhibitor, INI-43, on NFkB and AP-1 transcriptional function and inflammatory target gene expression. Furthermore, the dependence of cervical cancer cells on inflammatory transcription factor activity for their motility was monitored.

3.2 RESULTS

3.2.1. KPNB1 is necessary for NFkB cellular translocation

3.2.1.1. Monitoring the effects of KPNB1 inhibition on NFkB subcellular localisation by immunofluorescent analysis

To determine whether KPNB1 was required for NFkB translocation into the nucleus, the NFkB p65 subunit was monitored using immunofluorescence. HeLa cervical cancer cells were transfected with siRNA inhibiting KPNB1 after which they were stimulated with PMA to activate NFkB nuclear translocation. In control siRNA transfected cells stimulated with PMA p65 immunofluorescence appeared predominantly nuclear, while with KPNB1 inhibition, p65 fluorescence was predominantly cytoplasmic (Fig. 3.1 A). All images were quantified separating cells into two categories; 1) showing predominantly nuclear staining or 2) showing predominantly cytoplasmic staining and represented as a bar graph (Fig. 3.1 B). Approximately 250 cells per condition were scored and results show a substantive (81 %) retention of p65 staining in the cytoplasm of KPNB1-inhibited cells. Concurrently western blot analysis was performed and shows knockdown of KPNB1 in HeLa cells (Fig. 3.1 C). These results show that KPNB1 is necessary for NFkB nuclear localisation.

As further support and to monitor the effects of inhibiting nuclear transport via KPNB1 using a small molecule inhibitor, HeLa and SiHa cells were treated with INI-43. Results show that NFkB p65 is predominantly cytoplasmic in untreated cells while PMA stimulated the nuclear entry of NFkB. A pre-treatment with INI-43 was able to block nuclear entry of p65 in PMA treated HeLa cells (Fig. 3.2 A). For quantification purposes cells were also divided into the nuclear or cytoplasmic categories as before and represented as a bar graph (Fig. 3.2 B). A

cytoplasmic retention of 85 % of the p65 resulted following INI-43 treatment. Similar observations were made using SiHa cells treated with INI-43 where treatment with the INI-43 resulted in a perinuclear staining of p65 (Fig. 3.3 A). SiHa cells are smaller in size than HeLa cells and have a less distinct separation of nuclear and cytoplasmic regions when viewed under the microscope, thus presenting a more perinuclear p65 staining rather than cytoplasmic. Quantification of the nuclear or cytoplasmic categories are represented as a bar graph and show that INI-43 treatment caused 94 % of the p65 to be excluded from the nucleus in comparison to control untreated cells (Fig. 3.3 B). These results support that nuclear import associated with KPNB1 is required for the nuclear translocation of NFkB.

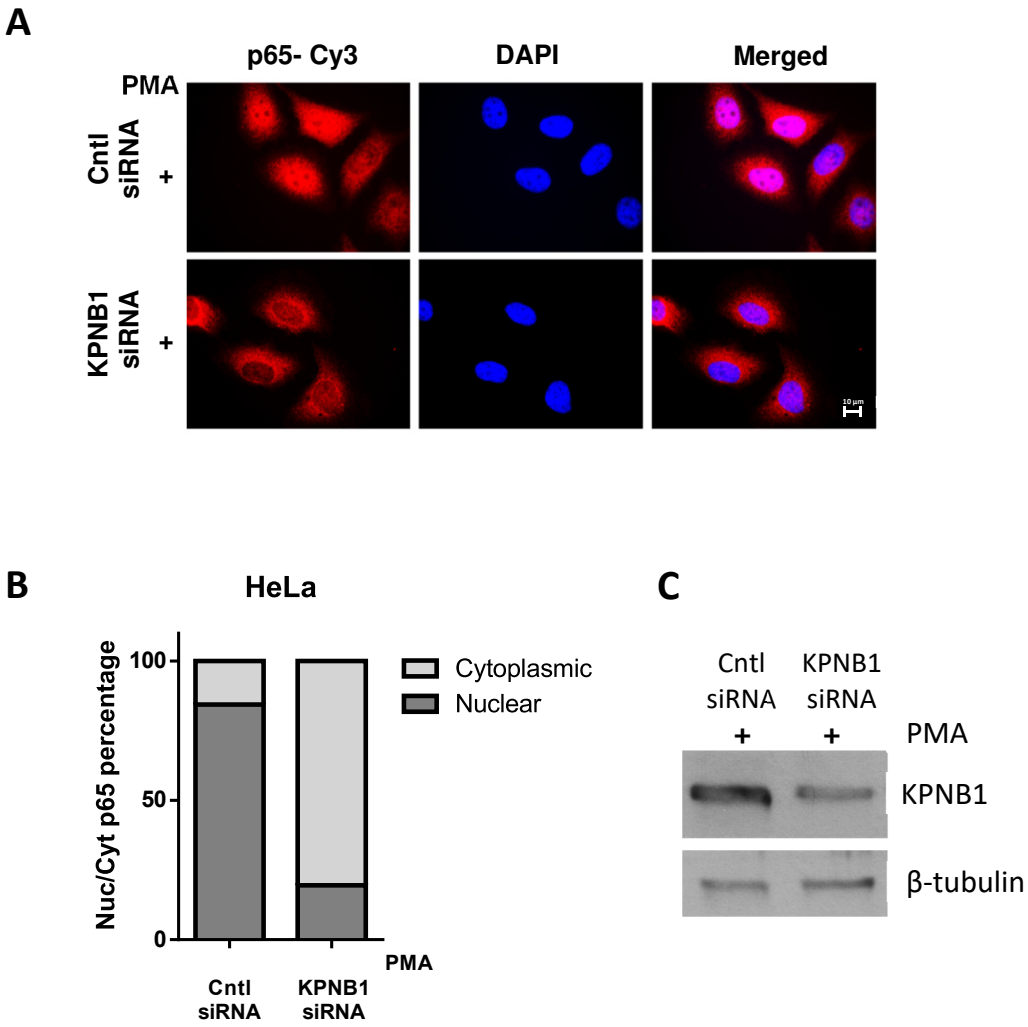


Figure 3.1: Effect of KPNB1 knockdown on NFkB p65 cellular localisation. A) Representative immunofluorescent images of HeLa cells transfected with control or KPNB1 siRNA and stimulated with 0.5 µM PMA for 1 hour, showing cellular localisation of Cy3-labelled NFkB p65 (red). DAPI (blue) was used as a nuclear stain. Scale bar= 10µm, magnification x1000. **B)** Immunofluorescent localisation of NFkB p65 was quantified, covering 250 cells over 25 images per treatment and categorized as showing "predominantly cytoplasmic" or "predominantly nuclear" staining. The graph was plotted using the percentage nuclear and cytoplasmic p65 fluorescence. **C)** Western blot confirming KPNB1 knockdown. Experiments were performed at least three independent times.

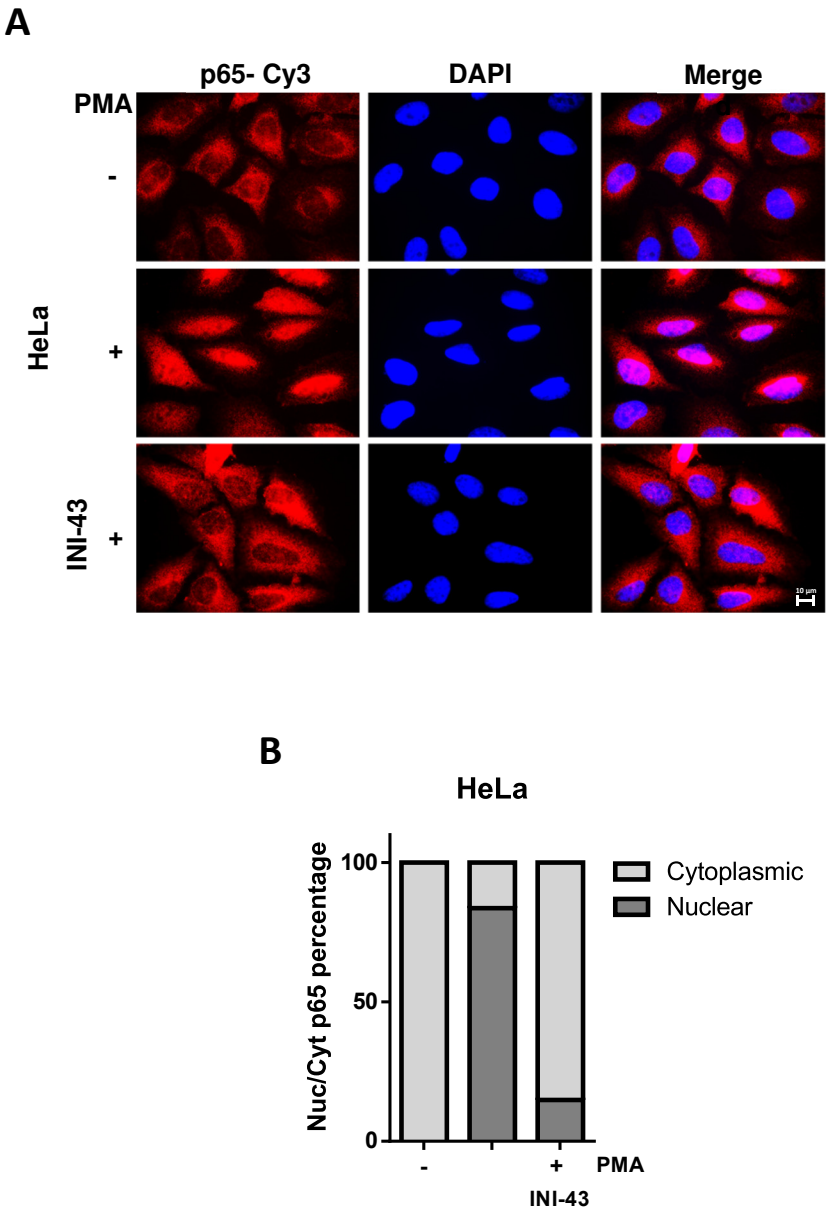


Figure 3.2: The effect of INI-43 on NFkB p65 cellular localisation in HeLa cells. A) HeLa cervical cancer cells were plated onto glass coverslips and pre-treated with 10 µM INI-43 for 2 hours and further stimulated with 0.5 µM PMA for an additional hour still in the presence of INI-43. NFkB p65 was Cy3-labelled (red) while nuclei were stained with DAPI (blue). **B)** Immunofluorescent localisation of NFkB p65 was quantified, covering 250 cells over 25 images per treatment and categorized as showing "predominantly cytoplasmic" or "predominantly nuclear" staining. The graph was plotted using the percentage nuclear and cytoplasmic p65 fluorescence. Scale bar= 10µm, magnification x1000. Experiments were performed at least three independent times.

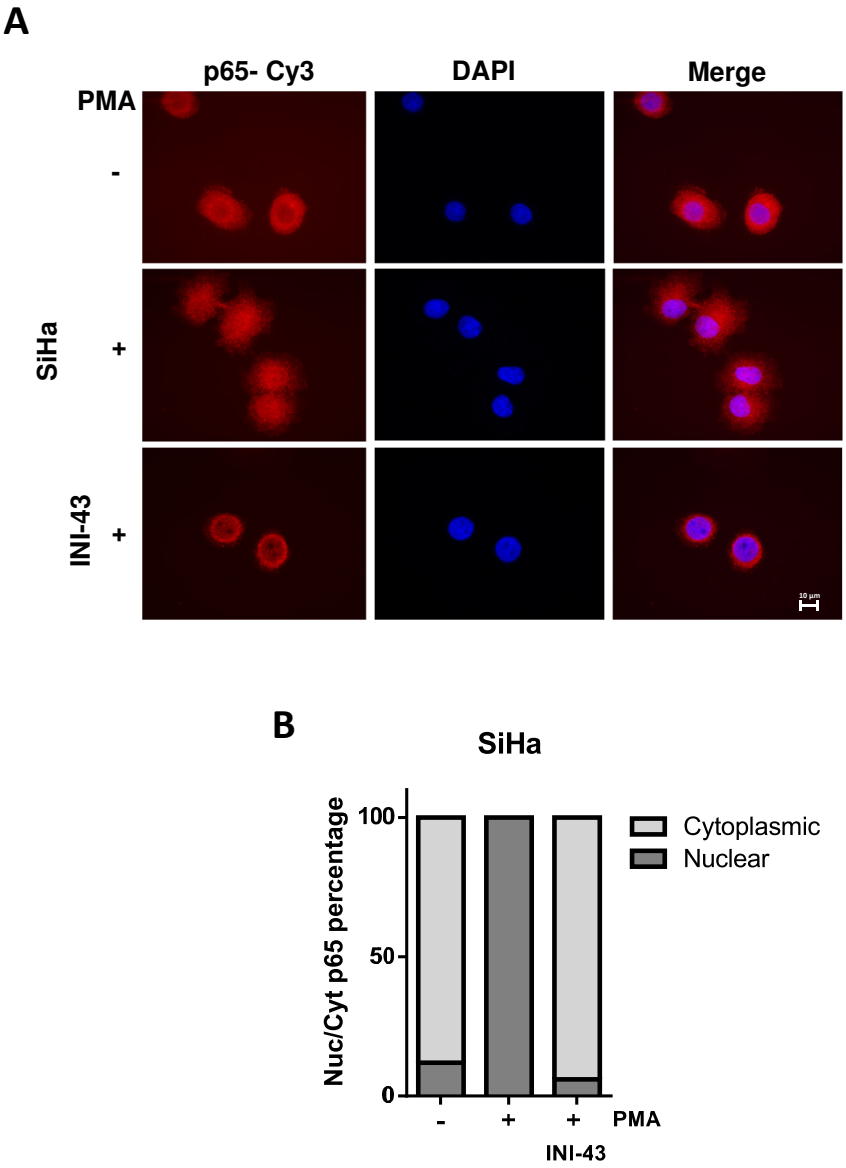


Figure 3.3: The effect of INI-43 on NFkB p65 cellular localisation in SiHa cells. A) SiHa cervical cancer cells were plated onto glass coverslips and pre-treated with 10 µM INI-43 for 2 hours and further stimulated with 0.5 µM PMA for an additional hour still in the presence of INI-43. NFkB p65 was Cy3-labelled (red) while nuclei were stained with DAPI (blue). **B)** Immunofluorescent localisation of NFkB p65 was quantified, covering 250 cells over 25 images per treatment and categorized as showing "predominantly cytoplasmic" or "predominantly nuclear" staining. The graph was plotted using the percentage nuclear and cytoplasmic p65 fluorescence. Scale bar= 10µm, magnification x1000. Experiments were performed at least three independent times.

3.2.1.2 Investigating the effects of KPNB1 inhibition on NFkB subcellular localisation using western blotting

Western blot analysis was used as an independent measure of NFkB subcellular localisation by fractionating cellular protein into cytoplasmic and nuclear fractions following nuclear import inhibition. HeLa cells transfected with KPNB1 siRNA showed reduced p65 and p50 protein levels in the nuclear fraction and increased protein levels in the cytoplasmic fraction, in comparison to the control (Fig. 3.4 A).

HeLa cells stimulated with PMA show an increase in nuclear p65 and p50 protein levels in comparison to unstimulated cells (Fig. 3.4 B). While, INI-43 pre-treatment resulted in reduced p65 and p50 protein levels in the nuclear fraction and increased protein levels in the cytoplasmic fraction. Similarly, PMA stimulation caused increased nuclear levels of p50 in SiHa cells while INI-43 treatment reduced both the nuclear and cytoplasmic protein levels of p50 (Fig. 3.4 C). These results support the requirement of KPNB1-mediated nuclear transport of NFkB in cervical cancer cells.

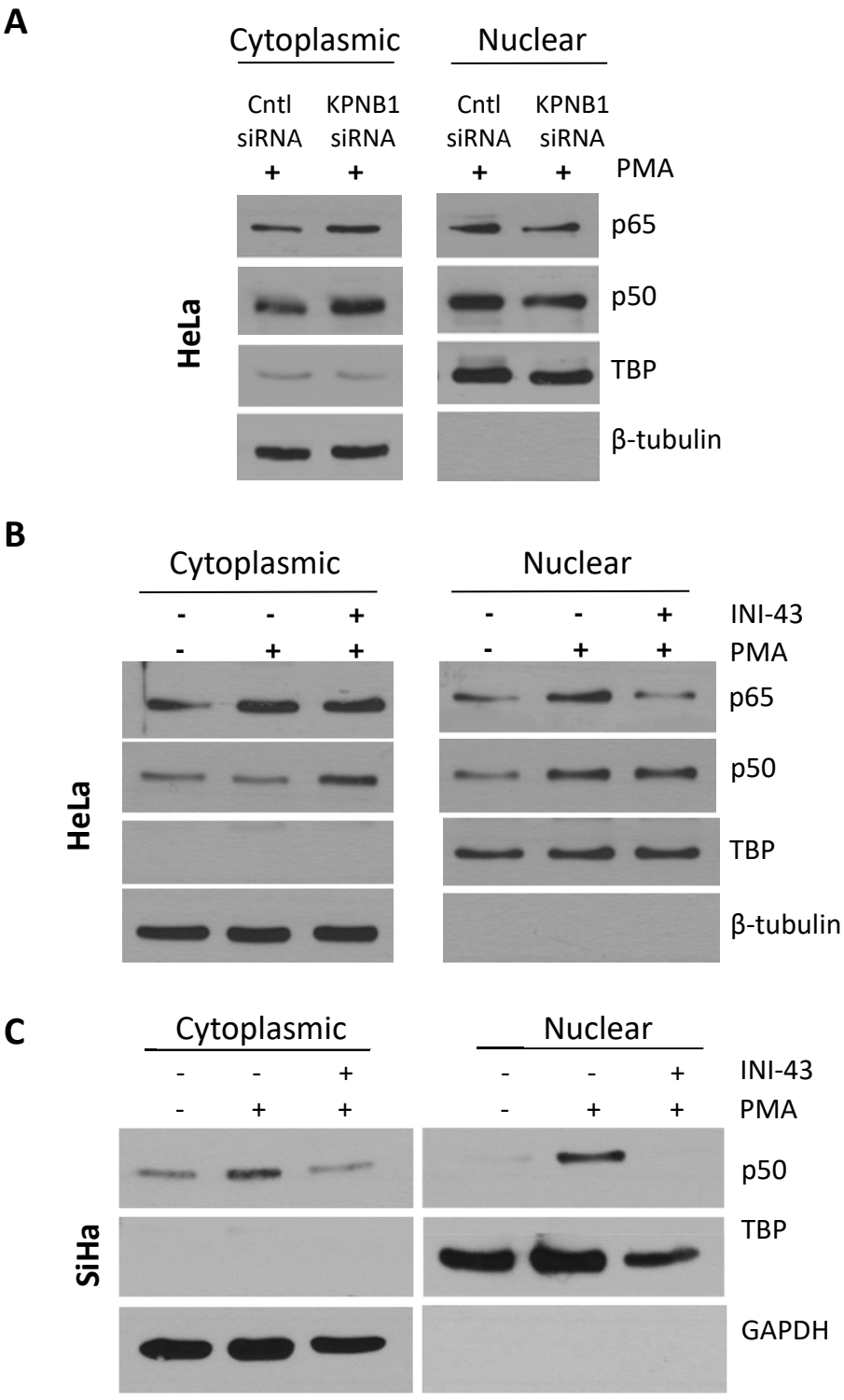


Figure 3.4: The effect of KPNB1 inhibition on nuclear entry of NFkB p65 and p50 in cervical cancer cells. A) Western blot analysis of NFκB p65 and p50 expression in the cytoplasmic and nuclear protein fraction of HeLa cells transfected with control and KPNB1 siRNA before a 1 hour 0.5 μM stimulation with PMA. **B)** Western blot analysis of HeLa cells following a 2 hour pre-treatment with INI-43 followed by a 1 hour 0.5 μM PMA stimulation showing NFkB p65 and p50 expression in the cytoplasmic and nuclear protein fractions. **C)** Western blot analysis of SiHa cells treated as per HeLa cells above showing NFkB p50 cellular localisation. β-tubulin or GAPDH were used as a cytoplasmic loading control while TBP (TATA-binding protein) was used as a nuclear loading control. Experiments were performed three independent times.

3.2.1.3 Effect of KPNB1 inhibition on NFkB in the nuclear cell fraction using electromobility shift assays

Electromobility shift assays were used to investigate NFkB binding to a NFkB consensus sequence in control and KPNB1-inhibited cells. Nuclear protein lysates from control and KPNB1-inhibited cells were incubated with an NFkB consensus sequence (5'-AGTTGAGGGGACTTTCCCAGGC-3'). A strong DNA-binding complex was detected in nuclear protein lysates from control siRNA transfected cells in the presence of PMA stimulation (Fig. 3.5 A). Complex formation was substantially reduced when KPNB1 was inhibited using siRNA. Western blotting was used to confirm protein nuclear/cytoplasmic fractionation and KPNB1 knockdown (Fig. 3.5 B).

INI-43 treatment similarly reduced DNA-protein complex formation in comparison to the PMA-stimulated control (Fig. 3.5 C). Supershift analysis was used to confirm p65 and p50 in the DNA-protein complex. For this, protein lysates were incubated with either the p65 or p50 antibody. Incubation with the p65 antibody in the binding reaction resulted in a supershifted complex indicating the presence of NFkB p65 (Fig. 3.5 D). A reduction in the binding when the supershift was performed using the NFkB p50 antibody suggests competitive binding of the protein with the biotin-labelled oligonucleotide. Together, these results confirm that KPNB1 is necessary for NFkB nuclear localisation and DNA-binding activity.

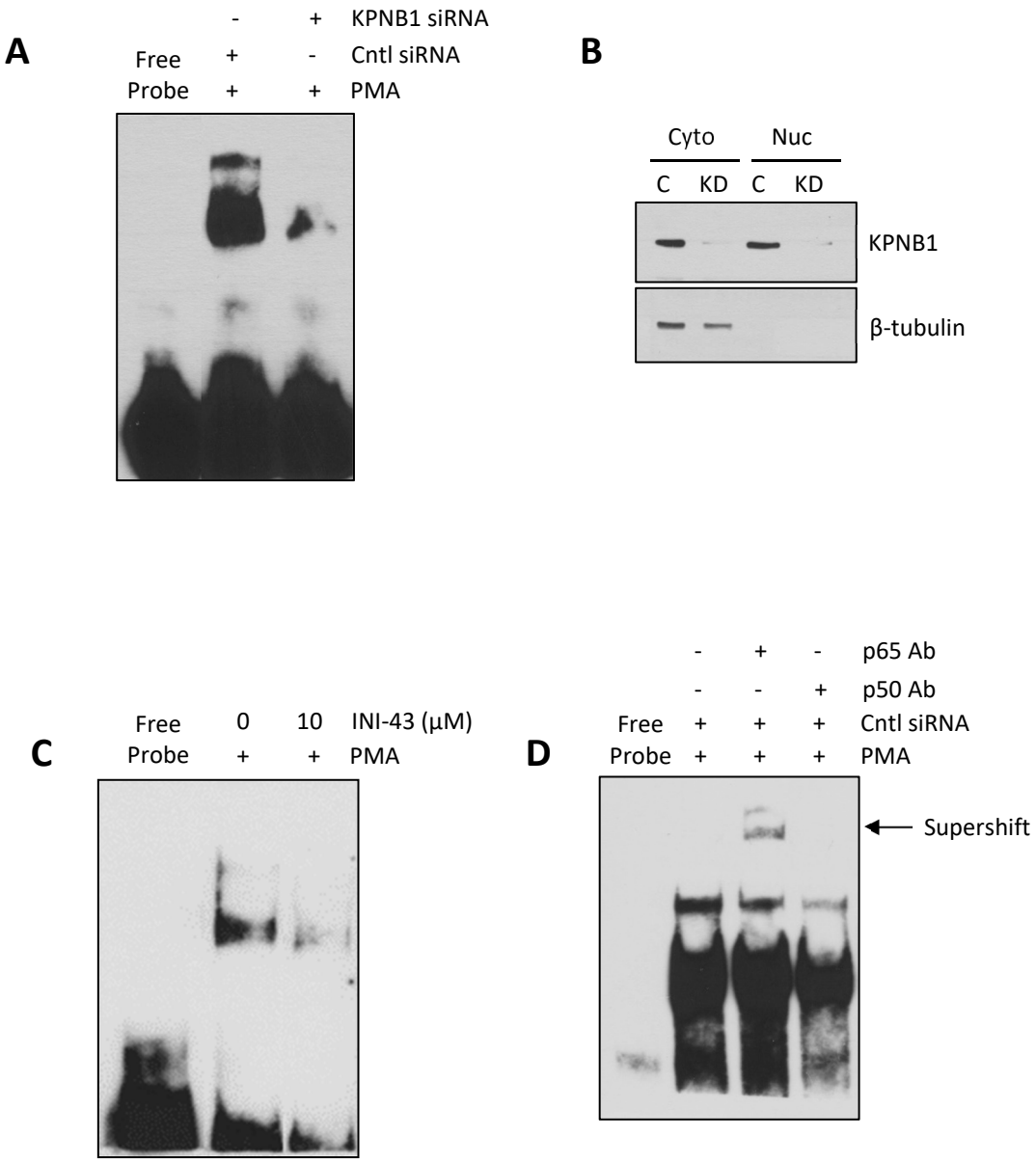


Figure 3.5: Formation of a nuclear NFkB/DNA binding complex following KPNB1 inhibition. A) Electromobility shift assay (EMSA) showing binding of nuclear protein to a biotin-labelled NFκB oligonucleotide following KPNB1 siRNA transfection in PMA-stimulated HeLa cells. **B)** Western blot analysis of fractionated protein samples confirmed KPNB1 knockdown and fractionation. **C)** EMSA of nuclear extracts obtained from cells pre-treated with 10 μM INI-43 for 2 hours followed by a 1 hour 0.5 μM PMA stimulation. **D)** Supershift analysis with the NFκB p65 antibody showing a shift in the DNA-protein complex while the p50 antibody reduced protein binding both confirming the presence of NFkB in the DNA-protein complex. Experiments were repeated two independent times.

3.2.2 The effect of KPNB1 inhibition on NFkB transcriptional activity

Our earlier results established that NFkB requires KPNB1 for its entry into the nucleus. By implication, then inhibiting KPNB1 should result in a change in NFkB transcriptional activity and transcriptional regulation of its target genes. The transcriptional activity of NFkB was monitored using a luciferase reporter construct containing an NFkB consensus binding site. KPNB1 inhibition using siRNA significantly reduced NFkB transcriptional activity using the NFkB promoter construct (Fig. 3.6 A). The effects of INI-43 and two commercially available nuclear import inhibitors of KPNB1, Importazole [195] and Ivermectin [193], on NFkB transcriptional activity was also measured following PMA stimulation. Nuclear import inhibitors were used at EC_{50} and half EC_{50} concentrations. PMA stimulation significantly increased NFkB transcriptional activity while pre-treatment with all three of the nuclear import inhibitors significantly reduced NFkB transcriptional activity (Fig. 3.6 B). Treatment of cells with a specific NFkB inhibitor, JSH-23, also showed a significant reduction in NFkB activity comparable to that of INI-43 (Fig. 3.6 C). A concentration of 20 μM JSH-23 was required to obtain a similar inhibition of NFkB transcriptional activity as 10 μM INI-43. This suggests that INI-43 is effective at inhibiting NFkB.

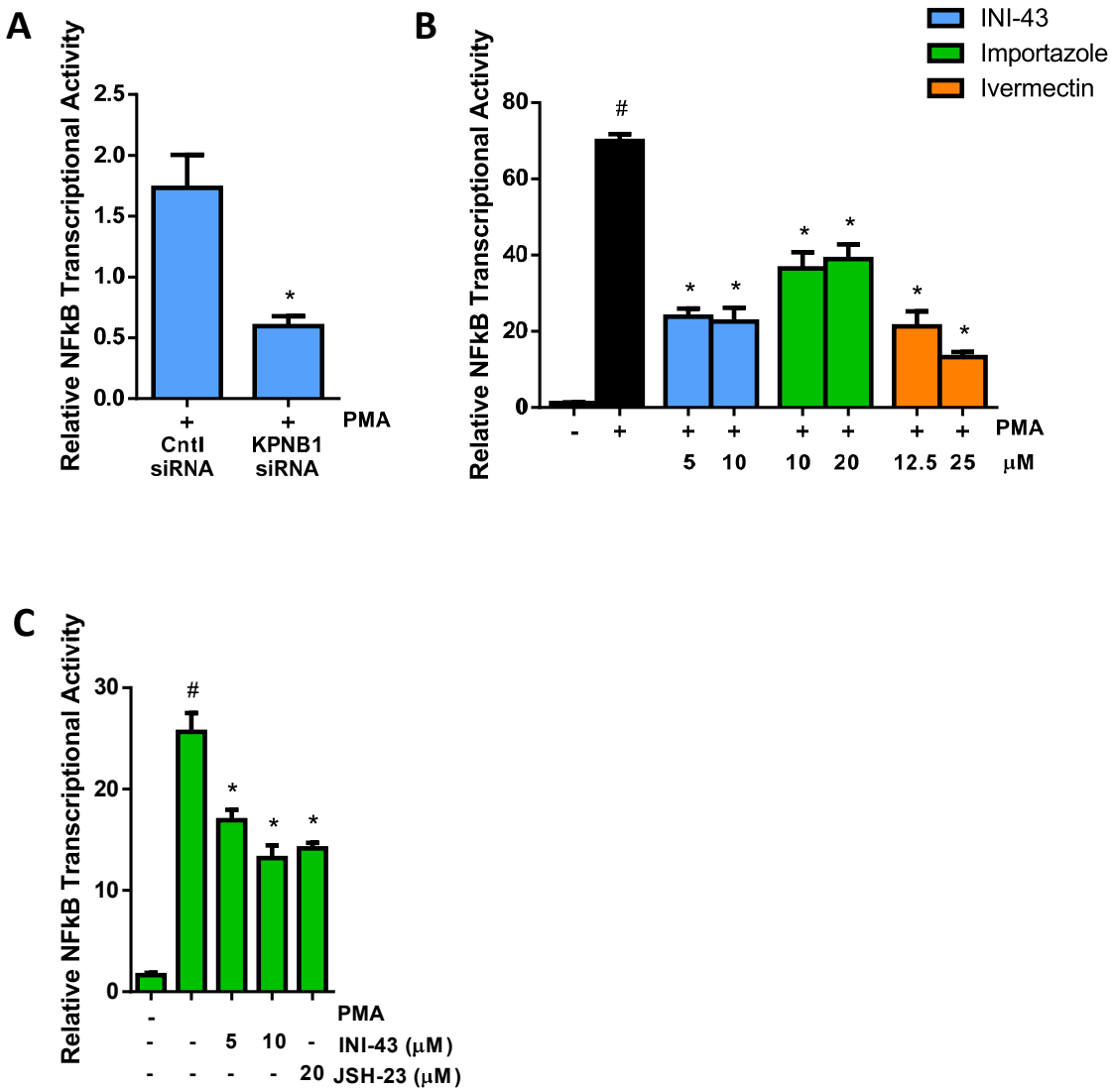


Figure 3.6: The effect of inhibiting nuclear import on the transcriptional activity of NFkB. A) NFκB transcriptional activity using an NFkB binding site-luciferase reporter transiently transfected into HeLa cells following KPNB1 knockdown and stimulation with PMA. **B)** NFkB transcriptional activity was measured following overnight pre-treatment with KPNB1 inhibitors; INI-43, Importazole and Ivermectin at 0.5x and 1x IC_{50} followed by a 3 hour 0.5 μM PMA treatment (24 hour total treatment). **C)** NFκB transcriptional activity in HeLa cells following a 21 hour pre-treatment with INI-43 or JSH-23 and a 3 hour 0.5 μM PMA stimulation (24 hr total treatment time). Results shown are the mean ± SEM of experiments performed in quadruplicate and repeated three independent times. ($^{\#}p<0.05$, significantly different from untreated control) (*$p<0.05$, significantly different from PMA-stimulated control)

3.2.3 The effect of KPNB1 inhibition on NFkB inflammatory target gene expression

Ultimately if NFkB transcriptional activity is affected this implications for NFkB target gene expression. NFkB plays a critical role in inflammatory signalling and therefore the target genes chosen for further investigation are inflammatory cytokines reported to be involved in cell biology changes associated with cancer. The expression of inflammatory cytokines; IL-1β, IL-6 and TNF-α were monitored in response to KPNB1 inhibition. HeLa cells transfected with KPNB1 siRNA showed reduced expression of the KPNB1 gene as well as significant reduction in mRNA expression of all three inflammatory cytokines (Fig. 3.7 A). The small molecule inhibitor, INI-43, had a similar inhibitory effect and significantly inhibited PMA-stimulated IL-1β, IL-6 and TNF-α gene expression which was comparable to the effect of the NFkB inhibitor, JSH-23 (Fig. 3.7 B, C & D). A significantly enhanced reduction of IL-1β and TNF-α mRNA expression in INI-43 treated cells in comparison to the effect of JSH-23 was observed. This suggests that INI-43 affects NFkB and other possible transcription factors that could regulate the expression of these cytokines (Fig. 3.7 B & D).

3.2.4 The effect of KPNB1 inhibition on AP-1 transcriptional activity

Having established that the inhibition of KPNB1 negatively affected NFkB activity we were interested in determining effects on another transcription factor, AP-1. The transcriptional activity of AP-1 was quantified using a luciferase reporter construct containing four AP-1 binding sites in a luciferase reporter construct. The effect of nuclear import inhibition using INI-43 as well as two other nuclear import inhibitors; Importazole and Ivermectin was assessed. PMA was used to stimulate AP-1 transcriptional activity (Fig. 3.8 A). Nuclear import inhibition by all three small molecules at their EC_{50} and half EC_{50} concentrations for 3 hours substantially decreased transcriptional activity in HeLa cells (Fig. 3.8 A). Similar results were

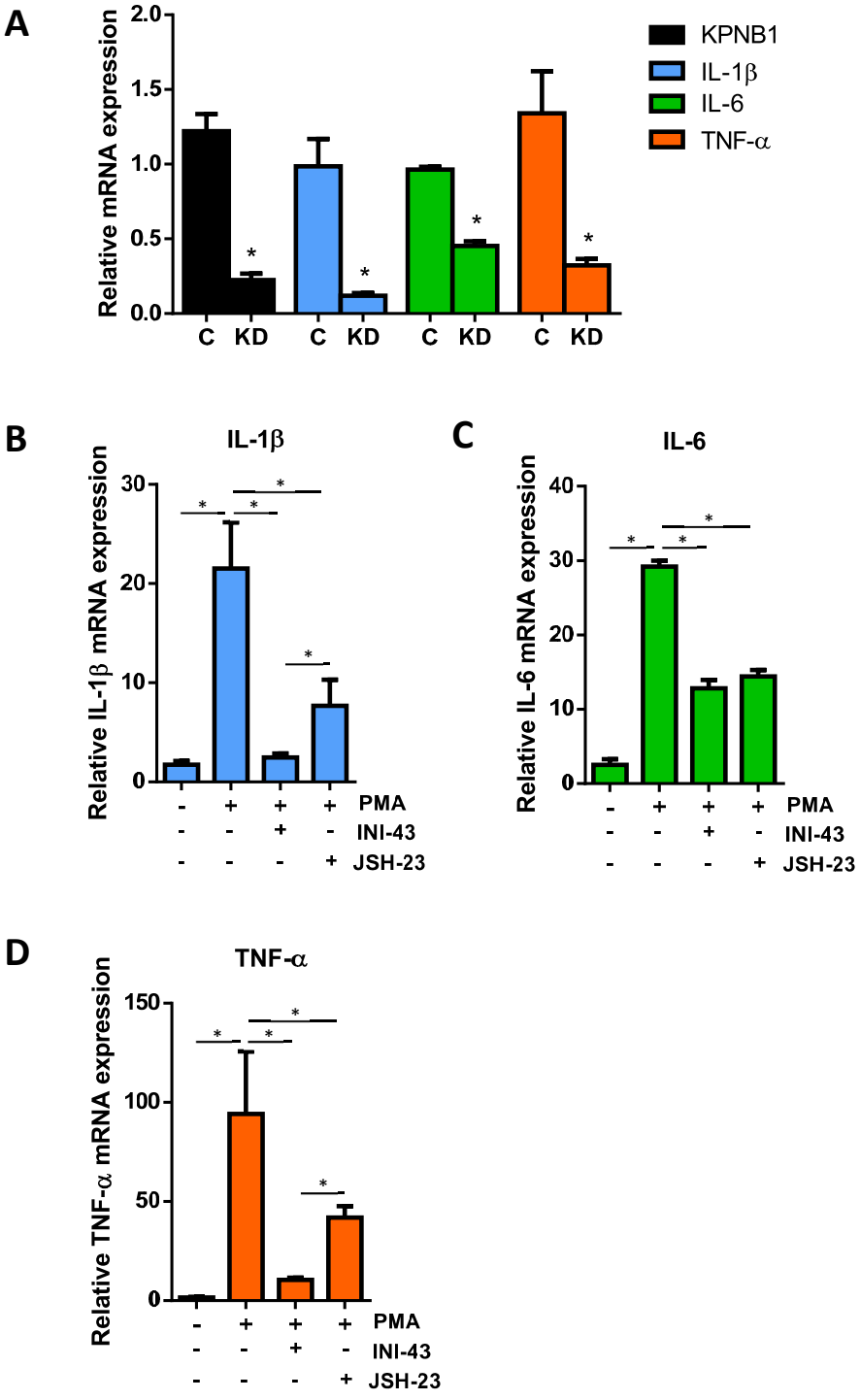


Figure 3.7: The effect of KPNB1 inhibition on NFkB inflammatory target gene expression. A) qRT-PCR analysis of mRNA expression showed the effect of KPNB1 knockdown (C= Control, KD= Knockdown) on KPNB1 mRNA expression, confirming knockdown, as well as expression of inflammatory target genes; IL-1β, IL-6 and TNF-α. **B)** IL-1β mRNA expression shown following stimulation for 1 hour with 0.5 µM PMA as well as pre-treatment for 2 hours with 10 µM INI-43 or 23 hours 20 µM JSH-23 followed by 1 hour PMA stimulation. The same conditions were used to look at mRNA expression IL-6 **(C)** and TNF-α **(D)**. Target gene expression was normalized to expression of the housekeeping gene, GusB. Results shown are the mean ± SEM of experiments performed in triplicate and repeated three independent times. (*p<0.05)

obtained in SiHa cells (Fig. 3.8 B). In SiHa cells we also showed that the inhibitory effect of INI-43 was comparable to that of a JNK inhibitor (SP600125), an upstream inhibitor of AP-1 (Fig. 3.8 B). For AP-1 to be active it needs to be located in the nucleus and its subunits phosphorylated by an upstream signalling kinase. For example, the c-JUN component can be phosphorylated by JNK and in turn become activated. We investigated whether inhibiting nuclear import via KPNB1 could affect the activation/ phosphorylation of c-JUN in a similar manner to that of a JNK inhibitor. Our results show that PMA was able to stimulate c-JUN phosphorylation i.e. AP-1 activation, and that treatment with INI-43 and SP600125 significantly reduced c-JUN phosphorylation (Fig. 3.9). These results support that INI-43 treatments interfere with AP-1 transcriptional activation.

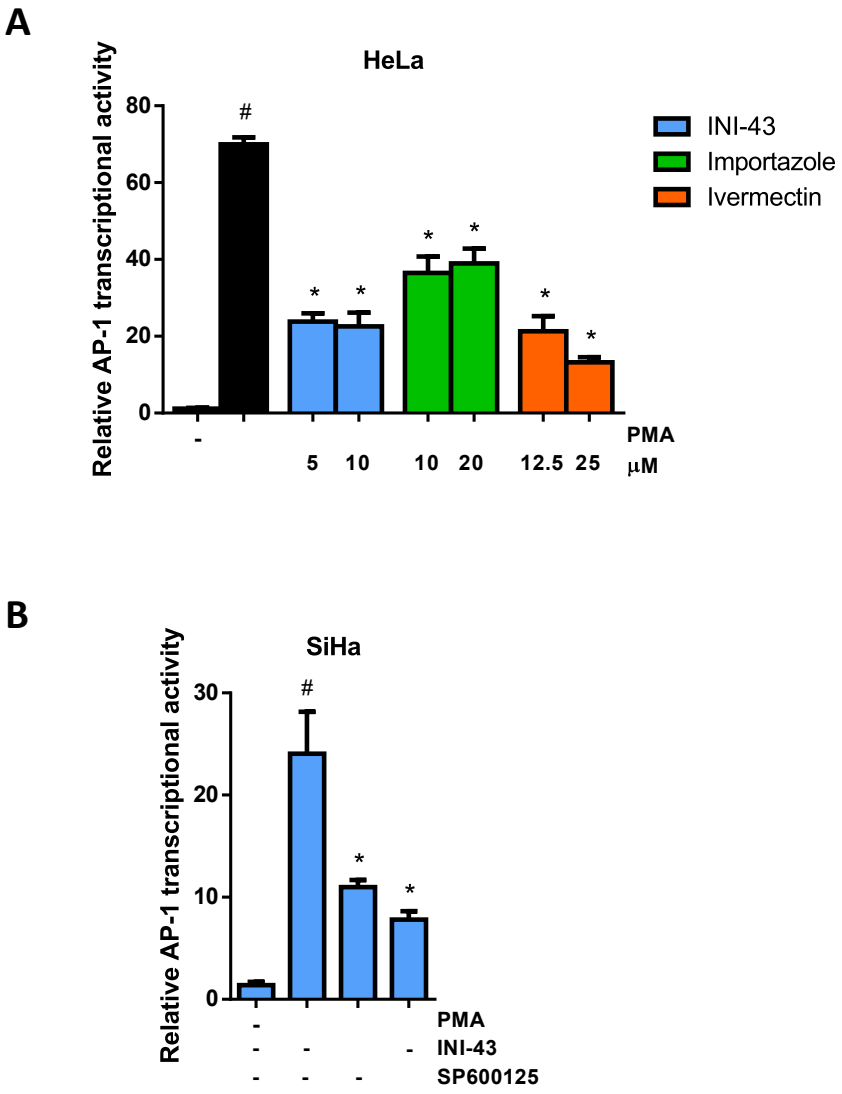


Figure 3.8: The effect of nuclear import inhibition on AP-1 transcriptional activity. A) AP-1 transcriptional activity in HeLa cells was measured using a luciferase reporter construct with multiple AP-1 binding sites following overnight pre-treatment with KPNB1 inhibitors; INI-43, Importazole and Ivermectin at 0.5x and 1x IC_{50} followed by a 3 hour 0.5 µM PMA treatment (24 hour total treatment). **B)** AP-1 transcriptional activity was also measured in SiHa cells following an overnight pre-treatment with 10 µM INI-43 or a 1 hour pre-treatment with SP600125 followed by a 3 hour 0.5 µM PMA stimulation. Results shown are the mean ± SEM of experiments performed in quadruplicate and repeated three independent times. ($^{\#}p<0.05$, significantly different from untreated control) (*$p<0.05$, significantly different from PMA-stimulated control)

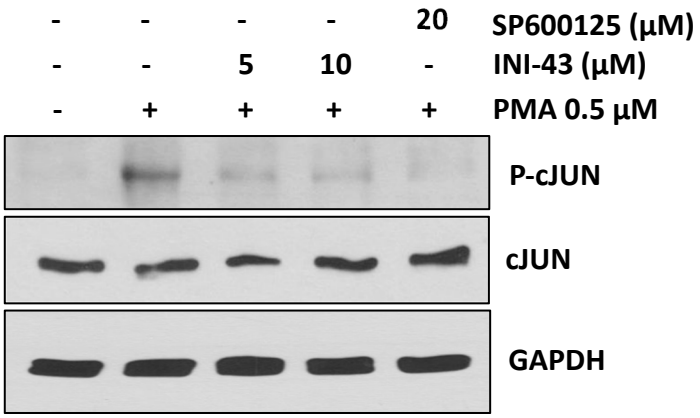


Figure 3.9: The effect of INI-43 on c-JUN phosphorylation in HeLa cells. Western blot analysis was used to determine the level of c-JUN phosphorylation following stimulation with 0.5 µM PMA and/or pre-treated with INI-43 or SP600125. GAPDH expression was used as a loading control. Western blot shown is a representative of three repeats.

3.2.5 The effect of KPNB1 inhibition on AP-1 inflammatory target gene expression

Having shown that inhibiting KPNB1-mediated functions with INI-43 blocks AP-1 transcriptional activity, we next investigated its effect on AP-1 target genes. The expression of AP-1 target genes; IL-6 and GM-CSF, associated with inflammation, were evaluated using qRT-PCR. Stimulation with PMA significantly increased mRNA expression of both genes while pre-treatment with INI-43 was able to reduce this expression (Fig. 3.10 A & B). As a positive control for AP-1 inhibition the JNK inhibitor, SP600125, an upstream inhibitor of the AP-1 signalling pathway was included. Activation of AP-1 requires that c-JUN be phosphorylated in the nucleus by phosphorylated JNK, although nuclear import of JNK is NLS-independent and therefore not reliant on KPNB1 [227, 228]. The enhanced inhibition by INI-43 on IL-6 expression in comparison to the effect of SP600125 (Fig. 3.10 A) again suggests that the effect of KPNB1 inhibition is mediated through its effect on a number of other transcription factors.

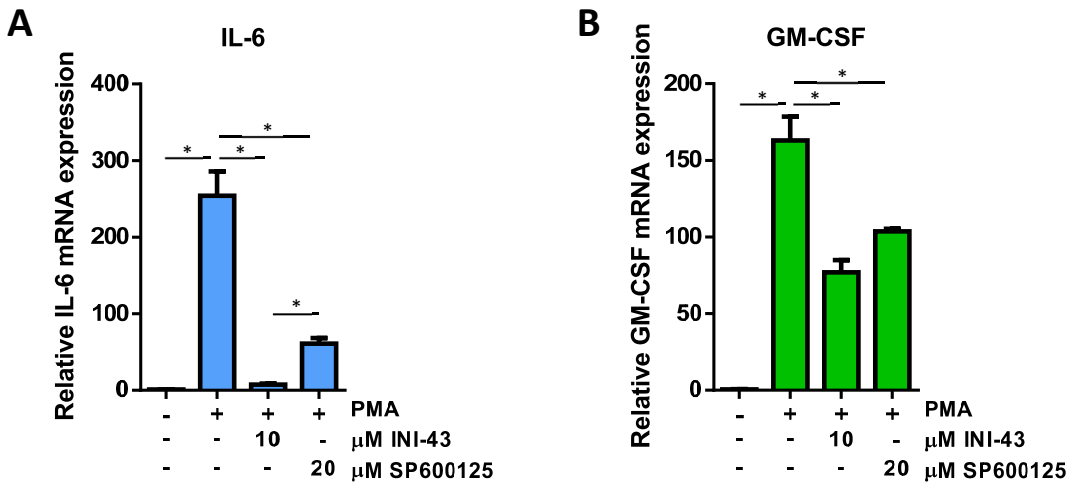


Figure 3.10: The effect of INI-43 on AP-1 target gene expression. qRT-PCR was used to measre the mRNA expression of AP-1 target genes; IL-6 **(A)** and GM-CSF **(B)**. HeLa cells were pre-treated with 10 µM INI-43 for 2 hours or 20 µM SP600125 for 1 hour followed by 0.5 µM PMA stimulation for an additional hour. Results shown are the mean ± SEM of experiments performed in triplicate repeated three independent times. (*p<0.05)

3.2.6 The effect of KPNB1 inhibition on IL-6 target gene activity and expression

The interleukin 6 (IL-6) inflammatory cytokine is a target gene of both NFkB and AP-1 transcription factors. Previous experiments showed that both the NFkB inhibitor, JSH-23, and the AP-1 inhibitor, SP600125, could significantly inhibit IL-6 mRNA expression. INI-43 treatment however, had a greater inhibitory effect on IL-6 mRNA expression. To further explore this, we next investigated the effect of KPNB1 inhibition on the IL-6 promoter.

3.2.6.1 The effect of KPNB1 inhibition on IL-6 promoter activity

The full-length IL-6 promoter has multiple transcription factor binding sites including NFkB and AP-1 binding sites both of which are important for inflammatory signalling (Fig. 3.11 A). We obtained the IL-6-PXP2 plasmid as a gift from Prof Luiz Zerbini, (ICGEB, University of Cape

Town) for investigation on whether KPNB1 inhibition could reduce IL-6 promoter activity given the effect we observed on NFkB and AP-1 transcriptional activity using artificial reporter constructs. Plasmid mapping was performed to confirm the IL-6 promoter construct. Digestion of the full-length plasmid with the restriction enzymes *BamH1* and *XHO1* released the IL-6 promoter insert of 1180 bps (Fig. 3.11 B). The insert was isolated and subjected to digestion by the *NLAIII* and *NEB4* restriction enzymes which revealed four fragments; 526 bps, 310 bps, 233 bps and 82 bps, perfectly matching the size of the fragments expected from the restriction map (Fig. 3.11 C). This served to confirm the identity of the IL-6-PXP2 promoter plasmid. The IL-6-PXP2 plasmid was transfected into HeLa cells and IL-6 promoter activity measured in control and KPNB1 inhibited cells. Both siRNA knockdown (Fig. 3.11 D) and INI-43 (Fig. 3.11 E) treatment had a significant inhibitory effect on IL-6 promoter activity. This data shows that inhibiting KPNB1 has an inhibitory effect on the activity of the IL-6 promoter which corresponds to the inability of the transcription factors, NFkB and AP-1, to enter the nucleus.

3.2.6.2 Investigating the effect of KPNB1 inhibition on IL-6 mRNA expression in cancer and non-cancer cells

The expression of the inflammatory cytokine IL-6 was investigated further in cervical cancer cell lines, HeLa, SiHa and CaSki, and a non-cancer fibroblast cell culture, FGo. INI-43 treatment significantly reduced PMA-stimulated IL-6 expression in HeLa, SiHa and CaSki cervical cancer cells (Fig. 3.12 A, B & C). However, INI-43 treatment had no effect on PMA-stimulated IL-6 expression in the non-cancer cells (Fig. 3.12 D). These results support the proposal that cancer cells are more sensitive to the effects of nuclear import inhibition via KPNB1 than non-cancer cells at the concentration of INI-43 used.

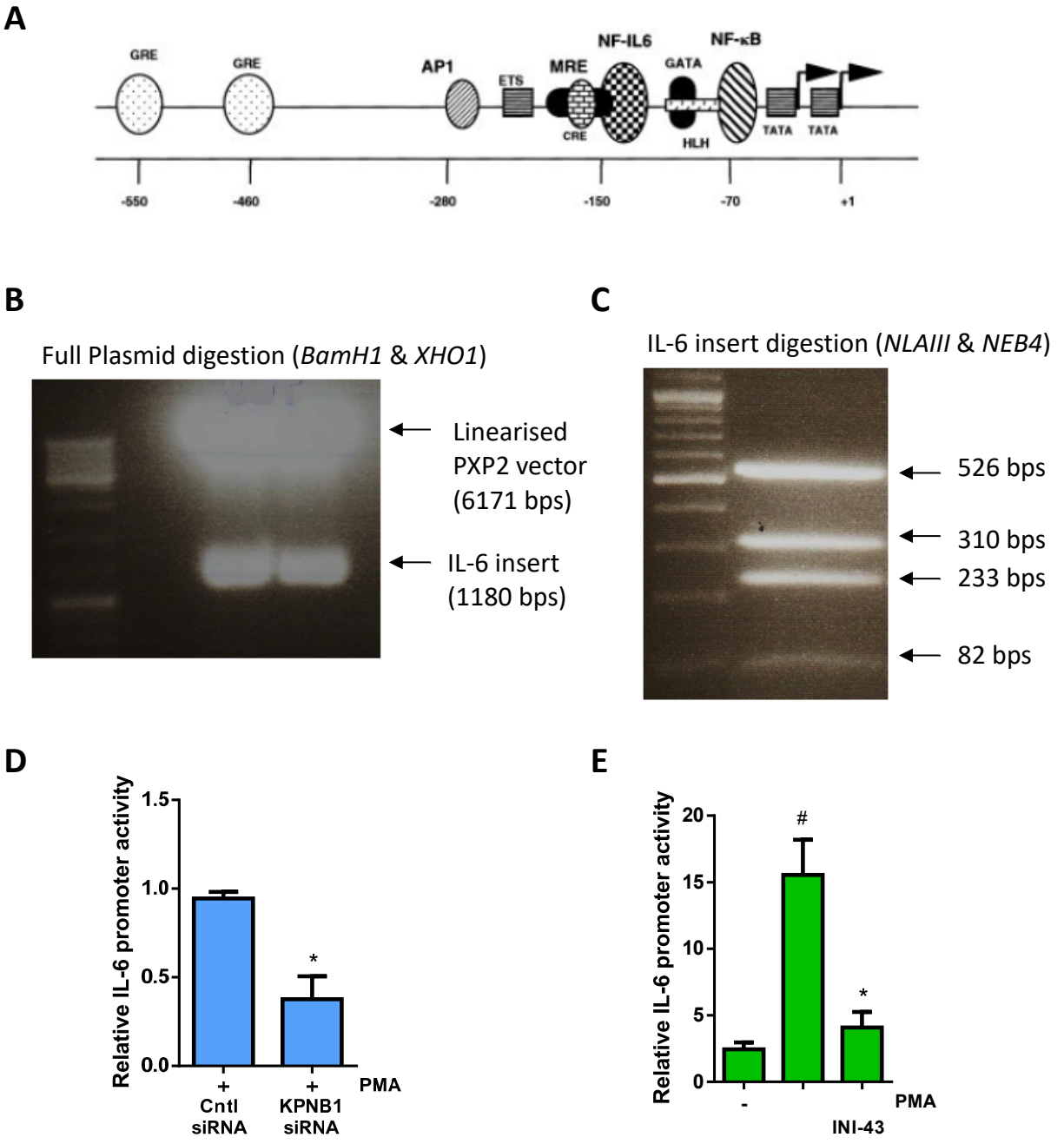


Figure 3.11: The effect of inhibiting KPNB1 on IL-6 promoter activity. A) A diagram showing the transcription factor binding sites on the full-length IL-6 promoter. **B)** The pXP2-IL-6 luciferase plasmid was digested using BamH1 and XHO1 to release the IL-6 promoter insert. Image shows agarose gel electrophoresis separating the linearized pXP2 vector (6171bps) and the IL-6 promoter insert (1180bps). **C)** Digestion of the IL-6 promoter insert by NLAIII and NEB4 showing four digestion products of sizes; 526 bps, 310 bps, 233 bps and 82 bps. **D)** IL-6 promoter activity is shown for HeLa cells transfected with control or KPNB1 siRNA using an IL-6 promoter luciferase reporter plasmid. **E)** IL-6 promoter activity shown for HeLa cells treated with 10 µM INI-43 for 21 hours followed by a 3 hour 0.5 µM PMA stimulation. Results shown are the mean ± SEM of experiments performed in quadruplicate and repeated three independent times. ($^{\#}p<0.05$, significantly different from untreated control) (*$p<0.05$, significantly different from PMA-stimulated control)

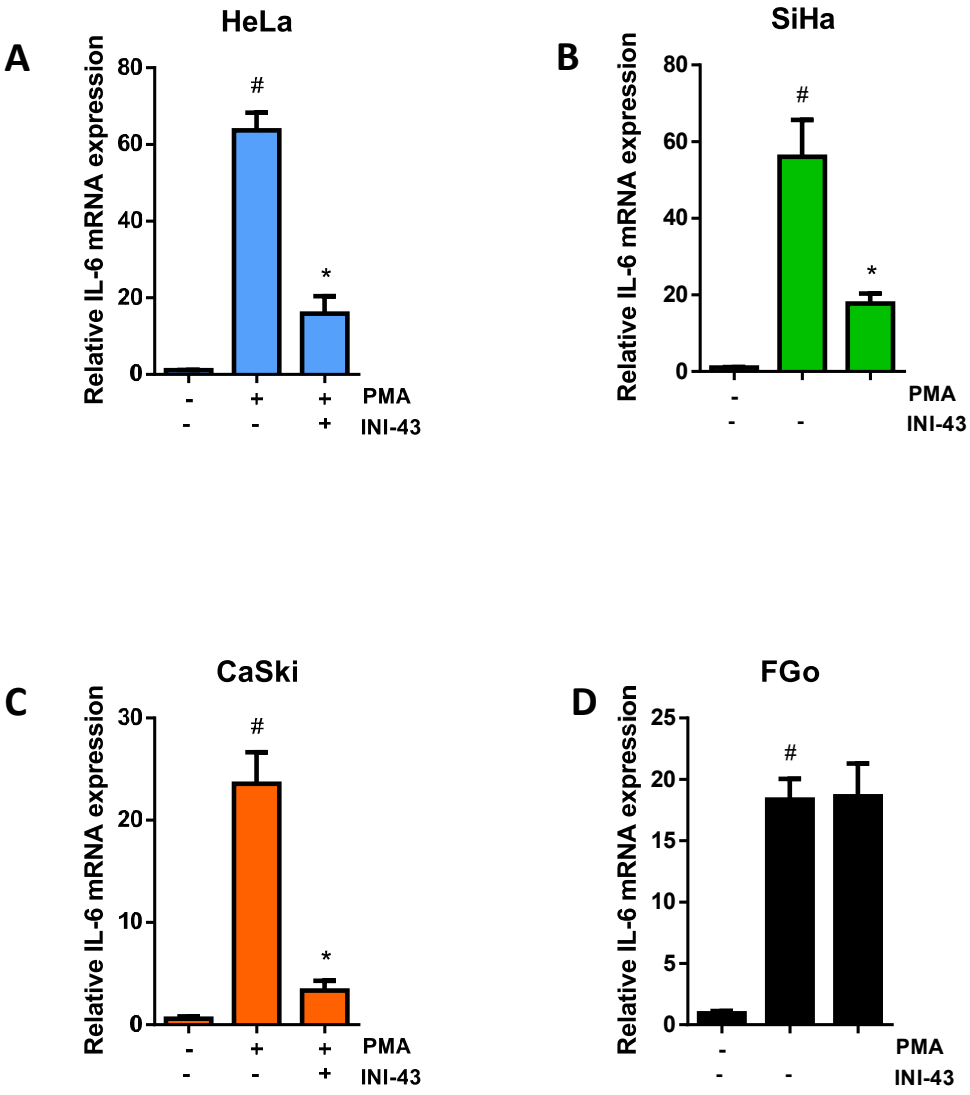


Figure 3.12: The effect of INI-43 on IL-6 mRNA expression in cervical cancer cells and normal fibroblasts. qRT-PCR was used to measure IL-6 mRNA expression in cervical cancer cells; HeLa **(A)**, SiHa **(B)**, and CaSki **(C)** and normal fibroblasts; FGo **(D)** after pre-treatment for 2 hours with 10 µM INI-43 followed by 1 hour 0.5 µM PMA stimulation. Results shown are the mean ± SEM of experiments performed in triplicate and repeated three independent times. ($^{\#}p<0.05$, significantly different from untreated control) (*$p<0.05$, significantly different from PMA-stimulated control)

3.2.6.3 The effect of INI-43 treatment on IL-6 protein expression in cervical cancer cells

We next monitored whether changes observed at the IL-6 mRNA levels translated to changes in the cytokine protein levels in cervical cancer cells. A Cytometric Bead Array was used to measure the concentration of secreted IL-6 present in the media of cervical cancer cell lines following INI-43 treatment. A standard curve was generated using known concentrations of IL-6 in order to quantify the IL-6 concentration in unknown samples (Fig. 3.13 A). Experiments were performed in triplicate with 300 events being recorded per sample. Treatment with INI-43 was able to significantly reduce PMA-stimulated IL-6 protein levels in HeLa and SiHa but not in CaSki cells (Fig. 3.13 B). The generally reduced secreted levels of IL-6 by CaSki cells, in comparison to that of HeLa and SiHa, could account for the inability to see an effect following INI-43 treatment.

3.2.7 Cervical cancer cell motility depends on inflammatory transcription factor activity

There is evidence in the literature that NFkB and AP-1 signalling contributes to cancer cell migration and invasion through the expression of inflammatory cytokines such as IL-6 and TNF-α [41]. We have shown above that NFkB and AP-1 signalling can be modulated through inhibiting the function of KPNB1. As a result of this we wanted to investigate whether the changes in cervical cancer cell motility seen when KPNB1 was inhibited are potentially attributed to the inhibition of NFkB and AP-1 signalling. In order to do this we performed a transwell migration assay where HeLa cells were treated with INI-43 alongside cells where NFkB was inhibited (JSH-23) as well as AP-1 signalling inhibited (SP600125). Inhibiting NFkB signalling showed a trend towards reducing HeLa cancer cell migration the results were not significantly different from the control (Fig. 3.14 A). Inhibiting KPNB1 using INI-43 and inhibiting AP-1 signalling using SP600125 however, reduced the migratory ability of cervical

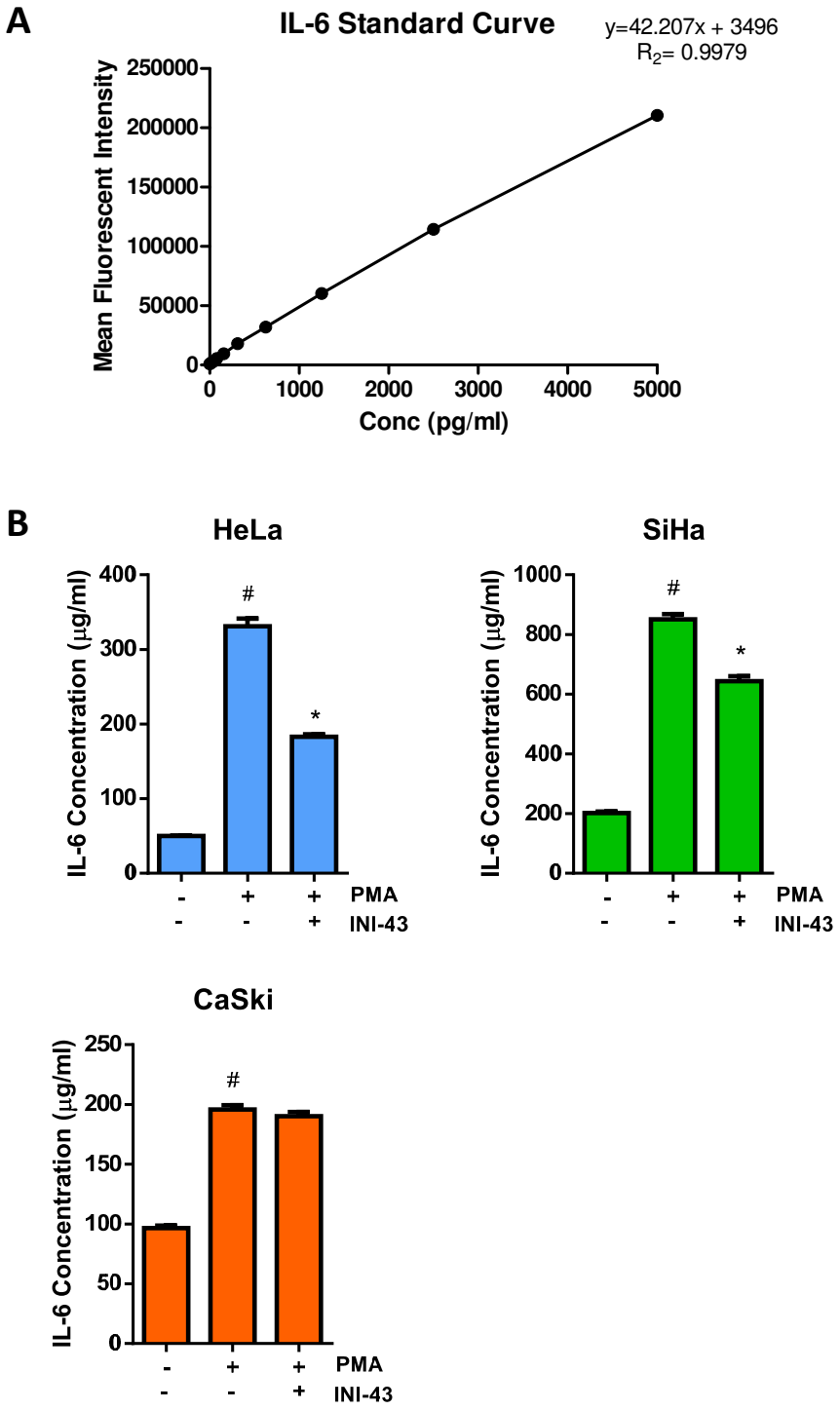


Figure 3.13: The effect of INI-43 on IL-6 protein expression in cervical cancer cells. A) For the Cytometric Bead Array samples of known IL-6 concentration were run on the BD Accuri C6 to create a standard curve from which samples of unknown IL-6 concentration could be calculated. **B)** Conditioned media was collected from cervical cancer cells overnight following a pre-treatment for 2 hours with 10 µM INI-43 and a 1 hour 0.5 µM PMA stimulation, the Cytometric Bead Array was used to determine the IL-6 concentration (µg/ml) in these samples. Results shown are the mean ± SEM of experiments performed in triplicate, recording 300 events per sample, and repeated twice ($^{\#}p<0.05$, significantly different from untreated control) (*$p<0.05$, significantly different from PMA-stimulated control)

cancer cells (Fig. 3.14 A). Quantification of the migration data is shown in Figure 3.14 (B). The data suggests that suppressed NFkB and AP-1 signalling following KPNB1 inhibition together contribute to the inhibitory effects on cancer cell biology.

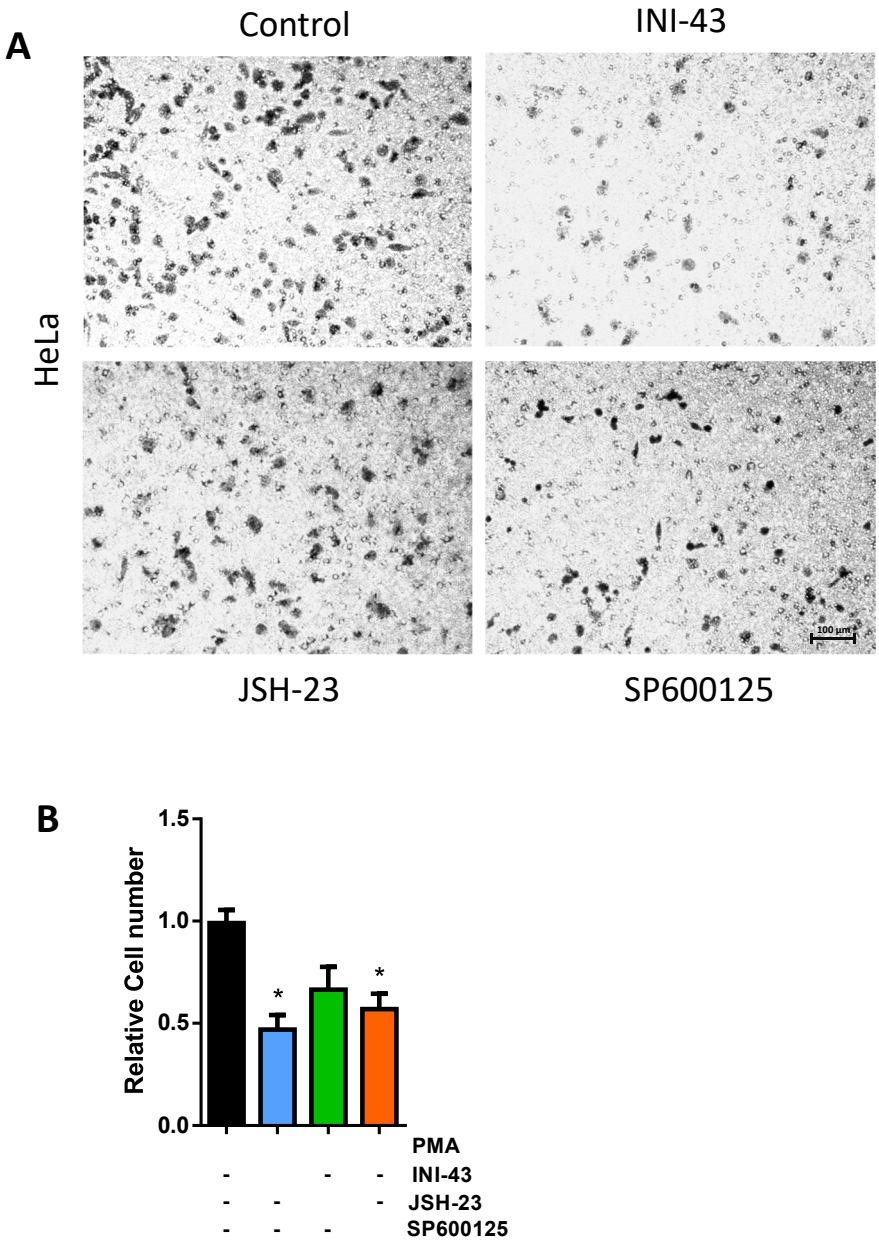


Figure 3.14: The role of transcription factors, NFkB and AP-1, in cervical cancer cell motility. A) Representative images of a transwell migration assay showing the amount of HeLa cells that were able to migrate through the membrane in the presence of 0.5 µM PMA (control) and following a 3 hour pre-treatment with 10 µM INI-43 and 20 µM JSH-23 and a 2 hour pre-treatment with 20 µM SP600125. Scale bar= 100µm, magnification x100. **B)** Quantification of the migration assay showing HeLa cell migration following INI-43, JSH-23 and SP600125 treatment, normalized to MTT cell viability. Results shown are the mean ± SEM of experiments performed in triplicate and repeated two independent times. (*p<0.05)

3.3 DISCUSSION

Our earlier data suggested that KPNB1 may play an important role in the survival as well as migratory and invasive potential of cervical cancer cells. A potential mechanism of action for the cell biology changes seen when KPNB1 is inhibited could relate to its function as a nuclear import protein. KPNB1 may be one of the key nuclear transporters of transcription factors associated with cancer cell growth, migration, invasion and metastasis.

NFkB and AP-1 are among the main transcription factors implicated in the development and maintenance of the hallmarks of cancer through the initiation of target gene expression [42, 88, 229]. As these transcription factors both require access to the nucleus in order to be functional we hypothesized that inhibiting nuclear import via KPNB1 may lead to their inactivity and ultimately various downstream effects on cancer cell biology. We identified KPNB1 to be required for the subcellular translocation and activity of the NFkB transcription factor. A study by Liang *et al.* (2013) found KPNB1 to be responsible for the cellular translocation of NFkB p65 and inhibiting its function resulted in the retention of NFkB p65 in the cytoplasm leading to reduced NFkB transcriptional activity [84]. Our data is in support of these findings. NFkB is an important mediator of inflammation while other transcription factors such as AP-1 have also been known to initiate expression of inflammatory cytokines [22, 62]. In this study we questioned whether inhibiting NFkB and AP-1 activity via inhibiting KPNB1 associated with the inhibition of inflammatory gene expression. KPNB1 was inhibited using two approaches; RNA interference and drug-mediated inhibition. The canonical NFkB pathway as well as c-JUN activation pathway was stimulated in cell culture using PMA which enhanced IL-1β, IL-6, TNF-α and GM-CSF expression. We show that stimulated cytokine expression could be blocked following inhibition of KPNB1 or through transcription factor specific inhibitors further confirming the reliance of NFkB and AP-1 activity on the nuclear

importer. NFkB and AP-1 both have a binding site on the IL-6 promoter and therefore both play a role in the initiation of IL-6 expression. The ability of both the NFkB inhibitor and the JNK inhibitor to block IL-6 mRNA expression confirms this. Stimulated IL-6 mRNA expression was inhibited by INI-43 treatment in all three cervical cancer cell lines but had no effect on the non-cancer cells used in our study. This suggests that the cancer cells are more sensitive to the inhibition of KPNB1, as was previously suggested in the proliferation studies. The inhibition of IL-6 expression in cancer cells was also seen at a protein level in HeLa and SiHa cells. In line with our findings, a study by Jin *et al*. (2012) showed that the alkaloid, Crytopleurine, was able to inhibit TNF-α, PMA or LPS stimulation of NFkB in breast cancer cells. Theses authors show that NFkB nuclear localisation and activity were both inhibited by Crytopleurine and caused reduced IL-6 and IL-1β target gene expression among others. Furthermore, they found inhibition of NFkB to inhibit MMP-9 expression and in turn reduce invasion of cancer cells using a transwell assay [230]. The pro-inflammatory cytokines; IL-1β, IL-6, TNF-α and GM-CSF have been implicated in cell biology changes required for tumour progression [46, 231-233]. These changes include increased migratory ability and invasive potential which are required for the epithelial-to-mesenchymal transition (EMT) that precedes metastasis [206]. On activation of the transcription factor, NFkB, target genes such as TNF-α and IL-6 can subsequently form a positive feedback loop further activating NFkB as well as other transcription factors such as AP-1 [74]. Together activation of these transcription factors has been associated with down-regulated E-cadherin expression and upregulation of matrix metalloprotease production ultimately promoting EMT [47, 73, 88]. Both transcription factors have been found to play a primary role in regulating MMP expression [40, 88, 104].

To identify whether either NFkB or AP-1 are required for the migration of cervical cancer cells, each transcription factor was individually inhibited alongside INI-43 and the effect on migration analysed. Although inhibition of the AP-1 signalling pathway was able to

significantly reduce migration, neither inhibition of AP-1 or NFkB on their own were able to reduce migratory potential of cancer cells to the extent of the nuclear import inhibitor. This suggests that the effects seen following KPNB1 inhibition may be mediated through more than one transcription factor signalling pathway.

Transcription factors of the FOS/JUN, CREB/ATF and NFkB families are known to act synergistically to significantly increase the expression of the same target gene. It has therefore been proposed that inhibiting more than one transcription factor acting synergistically may be a more appropriate approach when thinking about anticancer strategies [234]. Darnell (2002) proposed that inhibiting nuclear import proteins may be a way of specifically targeting multiple overactive transcription factors [23]. Our data provides evidence that supports Darnell's proposal, showing that inhibiting KPNB1 affects AP-1 and NFkB transcriptional activities required for cancer cell biology.

The first evidence of KPNB1 possibly being involved in inflammation was in a model of collagen-induced arthritis. Sun *et al.* (2016) showed that the anti-inflammatory effects were a result of nuclear import inhibition of IL-1β-induced STAT3 which in turn reduced the expression of IL-6 and MMP-1 leading to reduced invasion of fibroblast-like synoviocytes [235]. To our knowledge our study is a first to identify KPNB1 as a potential therapeutic target for inflammatory signalling in cancer associated with enhanced proliferation, motility and invasiveness. Much research has been done on targeting individual transcription factors as a chemotherapeutic approach. This approach is often associated with broad-range side effects given the diverse role of each transcription factor in normal cellular functioning. Cancer cells however, have been reported to become "addicted" and highly dependent on the activity of certain transcription factors or oncogenes, hence inhibiting their function is thought to affect cancer cells to a greater extent than non-cancer cells [236]. Ideally a targeted approach would

be required to limit such off-target effects. Targeting KPNB1 as a means of transcription factor inhibition may be a favourable approach in cancers where KPNB1 is overexpressed e.g. in cervical cancer tissue in comparison to normal tissue. We have previously shown that normal cells are less dependent on KPNB1 for proliferation and survival [128, 203]. These results provide evidence for the nuclear import protein, KPNB1, in supporting the biological phenotype of cancer cells and that inhibiting its expression and activity has multiple inhibitory effects on cancer cells. Given that KPNB1 cargo proteins range beyond NFkB and AP-1, it would be worth considering the effect of KPNB1 inhibition on the activity of tumour suppressor protein cargoes, from a personalised medicine point of view. To further characterise the role of the small molecule inhibitor of nuclear import, INI-43, as a potential chemotherapeutic, further investigation on its effects on tumour growth and development using *in vivo* models is needed.

CHAPTER 4

THE EFFECT OF INI-43 ON *IN VIVO* TUMOUR GROWTH AND MORPHOLOGY

4.1 INTRODUCTION

Extensive *in vitro* studies using cervical cancer cell lines in tissue culture has provided evidence for the role of KPNB1 as an anticancer target. Cancer cell proliferation, migration and invasion was inhibited following KPNB1-mediated nuclear import inhibition using both siRNA and small molecule approaches. This suggests that these effects are mediated through the inhibition of nuclear entry of transcription factors implicated with cancer cell biology such as; NFkB and AP-1. While yielding promising data, research using cell culture models have limitations as they do not take the tumour microenvironment and host interactions into account. Little is known regarding the *in vivo* effects of KPNB1 inhibition on tumour development. Our laboratory has made attempts to generate stable and inducible KPNB1 knockdown cells for investigation in *in vivo* models, with little success. The lack of generating KPNB1 knockdown cancer cells was likely due to leaky systems and the lethality of the knockdown in cancer cells. We previously showed that the inhibitory effects of INI-43 on nuclear import *in vitro* is likely through targeting KPNB1 as the effects of INI-43 on KPNB1 target proteins and cancer cell biology closely mimics those where KPNB1 was inhibited specifically through siRNA knockdown.

In this study, we aimed to investigate the effects of INI-43 treatment on tumour growth using an *in vivo* mouse xenograft model for cervical cancer. Along with the analysis of tumour

growth following INI-43 treatment it is of interest to investigate the effect of INI-43 on KPNB1 expression and localisation *in vivo* using immunohistochemical staining. Previously, INI-43 has been shown to alter the cellular localisation of KPNB1 *in vitro* by causing KPNB1 to be retained in the cytoplasm and on the perinuclear membrane as opposed to being located predominantly in the nucleus of untreated cells [203].

When solid tumours begin to grow and progress to a more invasive phenotype several characteristic changes occur. Initially cancer cells closely represent their tissue of origin but as they develop and grow they begin to lose their characteristic cellular architecture and organisation. Grading of cancer tumours is commonly used by pathologists to describe cellular appearance based on their differentiation state. A well-differentiated tumour closely represents the tissue of origin and contains recognisable structure and organisation. While poorly-differentiated tumours lose their characteristic phenotype and structure. Differentiation state correlates with proliferative and invasiveness of tumours [237]. As tumours grow their nutritional demands increase which stimulates the formation of stroma, a supportive network within the tumour. Stroma is produced in close relationship with host fibroblasts and consists of the extracellular matrix (ECM); composed of fibrous proteins such as collagen, proteoglycans and hyaluronic acid, and the stromal cells. These cells include the fibroblasts but also adipocytes and immune cells. The stromal matrix is also filled with various cytokines, chemokines, growth factors and ECM-modifying enzymes [238, 239]. These components of the stroma work together with the cancer cells to promote growth, angiogenesis and invasion and is therefore an essential part of a growing tumour [240-242]. A drug that disrupts the development of the stroma within tumours could have significant anticancer abilities [243].

In this chapter, an ectopic xenograft mouse model for cervical cancer will be used to investigate the effect of INI-43 on tumour growth and the generation of histological data of KPNB1 levels, Ki-67 levels and morphological changes in control and INI-43-treated tumour sections. Our data suggests that the inhibition of tumour growth following INI-43 treatment associates with changes in tumour tissue morphology and structure as well as expression of extracellular matrix components.

4.2 RESULTS

4.2.1 The effect of INI-43 on tumour growth in an ectopic xenograft mouse model

Karyopherin beta 1 has shown promise as an anticancer target in *in vitro* studies and to further investigate its potential in an *in vivo* system, an ectopic xenograft mouse model was used. Cytotoxicity studies were performed prior to this study and showed that mice tolerated a range of INI-43 treatment concentrations including 50 mg/kg, the concentration used in this study [203]. CaSki, cervical cancer cells, were subcutaneously injected into the hind flank of athymic nude mice and tumours allowed to develop. Treatment through intraperitoneal injection of the vehicle (DMSO) or 50 mg/kg INI-43 commenced once tumours were palpable. Injections were performed three times per week for the duration of 4 weeks. The tumour size was measured using calipers over the course of treatment and tumour volume calculated. Analysis of tumour volume showed that INI-43 treatment resulted in a significant reduction in tumour size compared to the controls (Fig. 4.1). This result provides additional support for INI-43 as a small molecule with anticancer activity.

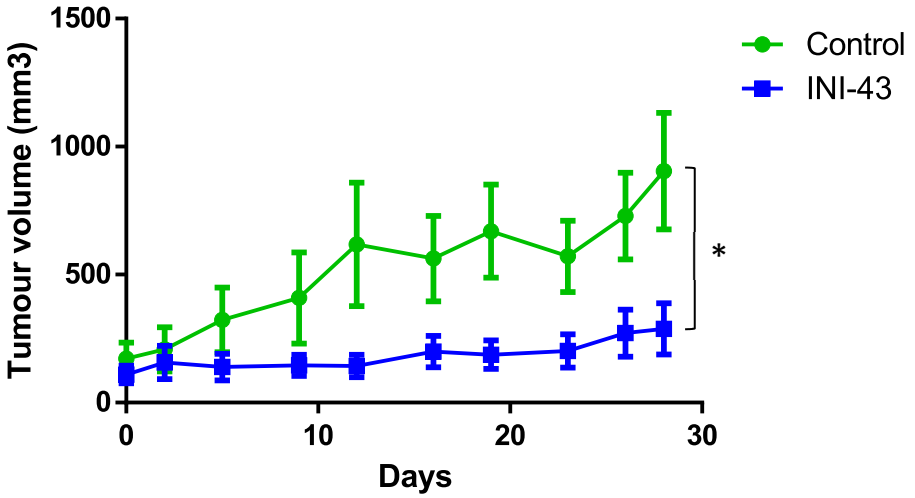


Figure 4.1: Tumour growth in an ectopic xenograft mouse model following INI-43 treatment. Tumour volume in control (DMSO) and INI-43 treated (50 mg/kg) nude mice bearing ectopic CaSki, cervical cancer cell, tumours. Results show mean ± SEM for 6 mice in each group. (*p<0.05).

4.2.2 The effect of INI-43 treatment on KPNB1 expression and localisation *in vivo*

At the end of the *in vivo* xenograft mouse study the tumours were removed for histological analysis. As INI-43 has been proposed to target KPNB1, it was of interest to investigate the expression and localisation of KPNB1 in the tumour samples following INI-43 treatment. Immunoperoxidase staining using an antibody specific for KPNB1 was used to analyse KPNB1 expression and localisation within the tumours. All immunohistological sections were 'blind' assessed and scored with assistance from an anatomical pathologist based in the Division of Anatomical Pathology at the University of Cape Town.

4.2.2.1 Expression of KPNB1 in INI-43-treated tumours

For immunohistochemical analysis of KPNB1 expression, human liver tissue was used as a positive control. Specificity of the antibody was confirmed with the negative control showing no immunoperoxidase (brown) staining and with only the haematoxylin (blue) counterstain visible while the positive control showed widespread immunoperoxidase staining (Fig. 4.2 A). KPNB1 has been known to be overexpressed in cervical cancer tissue in comparison to normal cervical tissue [128]. Here we support that data and show low KPNB1 expression seen in normal human endocervical tissue (Fig. 4.2 B). In comparison, untreated cervical cancer tumour samples from the xenograft mouse study showed substantially high levels of KPNB1 expression (Fig. 4.2 C). Sections from INI-43-treated samples, in contrast, showed a considerably reduced KPNB1 staining (Fig. 4.2 C).

4.2.2.2 Cellular localisation of KPNB1 within INI-43-treated tumours

Previously, using *in vitro* analysis we observed that KPNB1 showed predominantly nuclear localisation in untreated cervical cancer cells but following INI-43 treatment KPNB1

localisation was more cytoplasmic and in the perinuclear space [203]. Immunohistological sections were thus examined to determine KPNB1 expression in the nuclear and cytoplasmic compartments in sections from control and INI-43-treated tumours. The percentage of tissue showing either weak, moderate or strong KPNB1 staining was determined and the average percentage tabulated in Table 4.1. The results show that INI-43-treated tumour cells had reduced cytoplasmic KPNB1 expression levels (100% of cells showed weaker staining) compared to control tumours showing 60% strong cytoplasmic staining and 40% weak staining. Nuclear expression levels of KPNB1 remained largely unchanged. Also noted was that normal endocervical tissue showed exclusively nuclear KPNB1 expression while the cervical cancer tumours showed both nuclear and cytoplasmic expression (Fig. 4.2 B, C & Table 4.1).

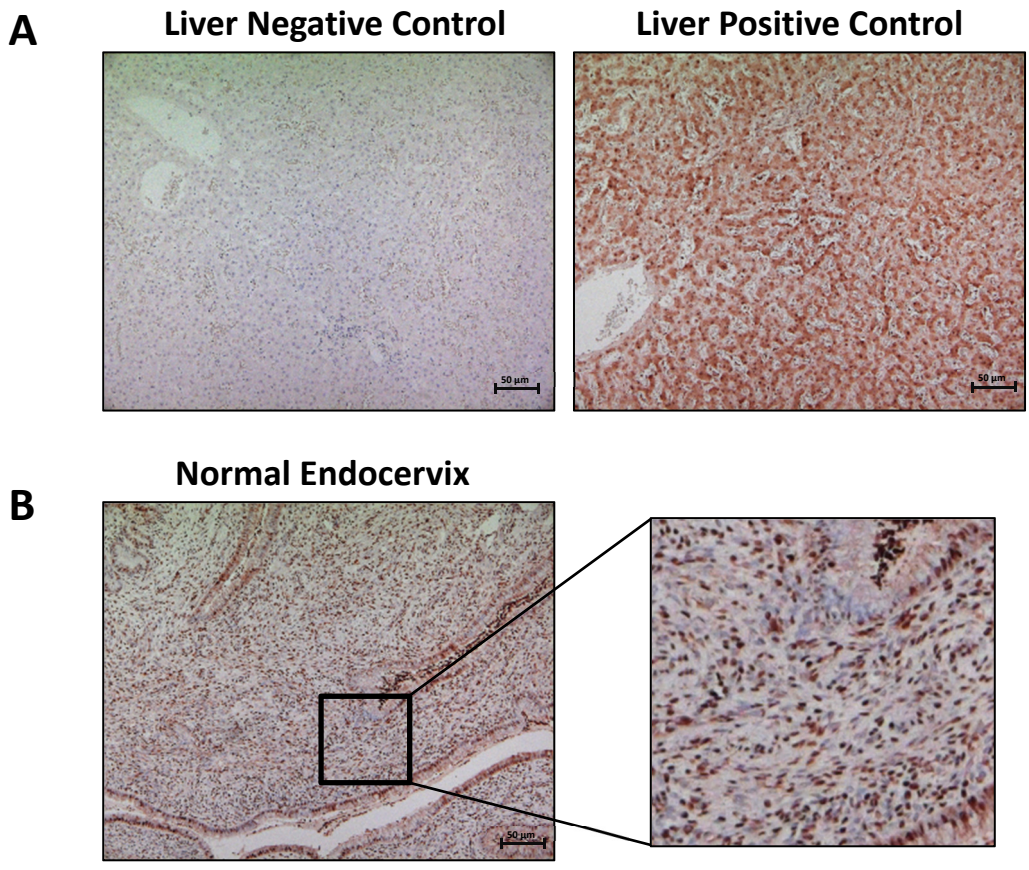


Figure 4.2 A & B: Immunohistochemical analysis of KPNB1 expression in INI-43-treated tumours. A) Negative and positive controls in human liver tissue samples for KPNB1 immunoperoxidase staining (brown), counterstained with Haemotoxylin (blue). **B)** KPNB1 staining in normal human endocervix tissue, inset showing zoomed view of nuclear stain. Scale bar = 50 µm, magnification x200.

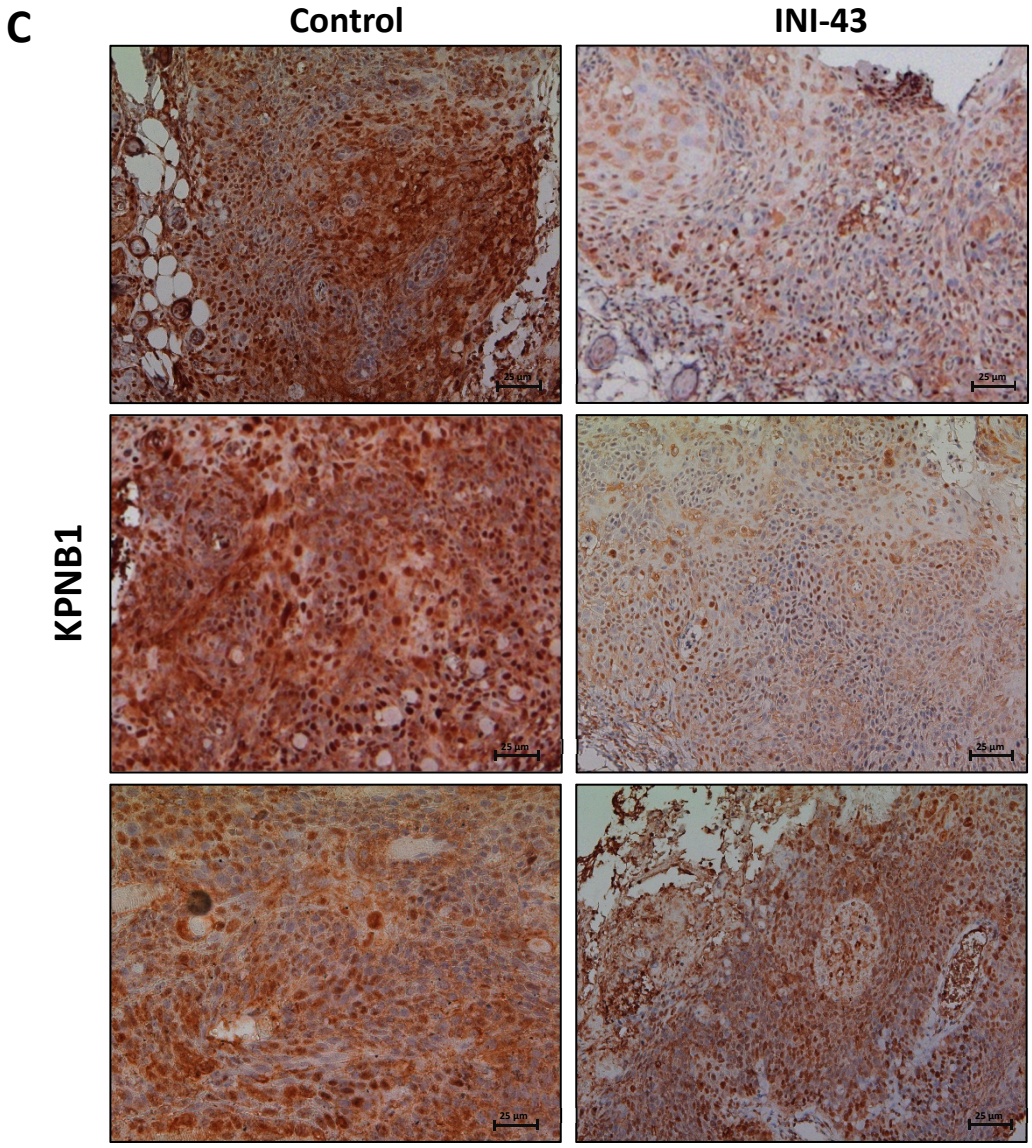


Figure 4.2 C: Immunohistochemical analysis of KPNB1 expression in INI-43-treated tumours. Representative images of KPNB1 staining in untreated control (DMSO) and INI-43-treated (50 mg/kg) CaSki xenograft tumours. Results shown represent tumour samples from 3 mice per treatment. Scale bar= 25 µm, magnification x400.

TABLE 4.1: Cellular expression of KPNB1 following INI-43 treatment *in vivo*

			Treatment (% tissue)		
	KPNB1 expression	**Endocervix**	**Control**	**INI-43**	**Effect**
Nuclear Intensity					
	Weak (+)	0	0	0	
	Moderate (++)	0	100	100	Unchanged
	Strong (+++)	100	0	0	
Cytoplasmic Intensity					
	Weak (+)	0	40	100	↑
	Moderate (++)	0	0	0	
	Strong (+++)	0	60	0	↓

To further investigate the localisation of KPNB1 in the tumours, the percentage of cells that showed nuclear versus cytoplasmic expression of KPNB1 was assessed. KPNB1 showed a greater cytoplasmic localisation and less nuclear staining in INI-43-treated tumour cells (Fig. 4.3 A & B). Fewer cells in the INI-43-treated tumours showed nuclear KPNB1 staining with more cells showing cytoplasmic KPNB1 localisation of which the cytoplasmic KPNB1 expression levels were considerably reduced. Together with the previous results, nuclear KPNB1 expression levels remain unchanged however fewer cells within the tumour showed nuclear KPNB1 staining. Reciprocally more cells showed cytoplasmic KPNB1 localisation although the cytoplasmic KPNB1 expression levels were considerably reduced.

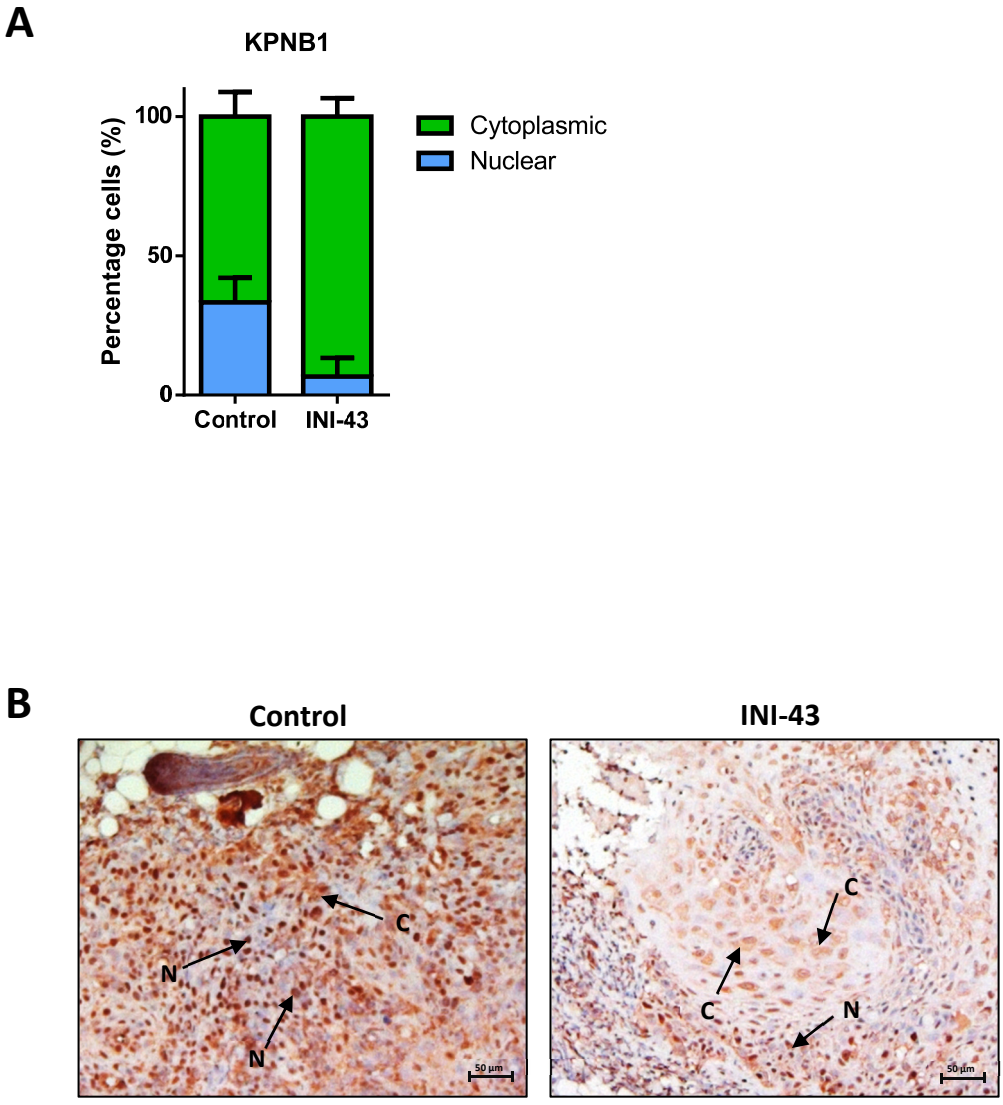


Figure 4.3: KPNB1 cellular distribution within INI-43-treated tumours. A) Bar graph showing the average percentage of cells with cytoplasmic or nuclear KPNB1 staining across 3 tumours per treatment shown as the mean ± SEM. **B)** Representative images showing immunoperoxidase staining for KPNB1 with arrows indicating nuclear (N) and cytoplasmic (C) staining for control and INI-43-treated tumours. Scale bar= 50 µm, magnification x200.

4.2.3 The effect of INI-43 on cellular proliferation of *in vivo* tumours

Nuclear import inhibition using INI-43 could significantly reduce tumour growth *in vivo* as well as have cytotoxic effects *in vitro*. These results lead us to investigate the effect of INI-43 on proliferation of cells within the xenograft tumours. Immunoperoxidase staining for the marker of proliferation, Ki-67, was used to detect proliferating cells within the tumours. As controls for Ki-67 staining, tissue of the human appendix that contains actively proliferating cells was used as a positive control and a negative control which showed no immunoperoxidase staining and only the haematoxylin counterstain (Fig. 4.4 A). Results showed considerably fewer cells stained positive for Ki-67 in the INI-43-treated tumours in comparison to the control (Fig. 4.4 B). The reduced proliferation of INI-43-treated tumours is in support of our previous results which show reduced tumour size in mice following INI-43 treatment. In addition, it was noted that Ki-67 staining appeared negative in regions of well-differentiated tissue and positive in regions of poorly-differentiated tissue and in the basal cell layer (Fig. 4.4 C).

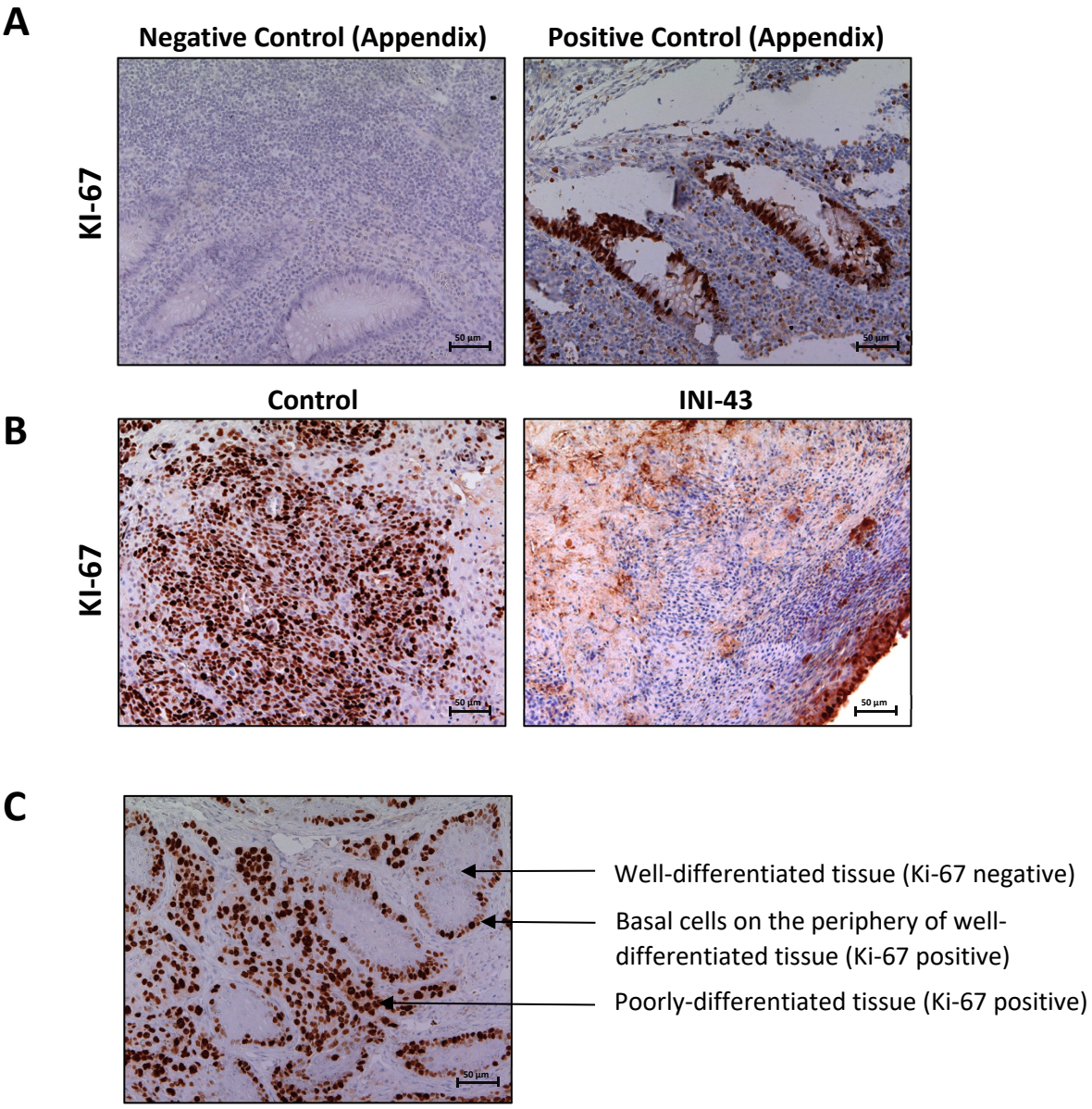


Figure 4.4: Assessment of proliferation following INI-43 treatment *in vivo*. A) Images representative of the negative and positive controls for Ki-67 immunoperoxidase staining in human appendix tissue samples, counterstained with Haemotoxylin. **B)** Representative images of Ki-67 staining in control untreated and INI-43-treated tumours. **C)** A representative image of differential Ki-67 staining showing positive staining in poorly-differentiated tissue and the basal cell layer and negative staining in areas of well-differentiated tissue. Scale bar= 50 µm, magnification x200.

4.2.4 Monitoring histological changes in tumours treated with INI-43

4.2.4.1 The effect of INI-43 on differentiation status

The correlation between positive Ki-67 staining and poor tissue differentiation in the previous result is to be expected as poorly-differentiated tissue is characteristically invasive and actively proliferating while the inverse is true for well-differentiated tissue. The reduced presence of proliferating cells after treatment with INI-43, as determined through Ki-67 immunostaining, lead us to further investigate the differentiation status of tissue within the tumours. Tissue differentiation is generally used as a visual means to grade cancer tumours. Well-differentiated tissue closely represents the tissue of origin, with the cells appearing large and eosinophilic with well-defined nucleoli. The cells are also layered in a uniform architectural pattern and generally grow at a slower rate. In contrast, poorly-differentiated tissue lose most of their epithelial characteristics and architecture. The cells contain poorly-defined nucleoli and have enlarged or even multiple nuclei. Poorly-differentiated tissue tends to grow and spread more quickly. H&E staining of the tumour sections allowed us to distinguish between well- and poorly-differentiated tissue. INI-43-treated tumours showed a trend towards containing more well-differentiated tissue and less poorly-differentiated tissue than the control untreated tumours (Fig. 4.5 A). The percentage of tissue that was well- versus poorly-differentiated in all the control and treated tumours was quantified and shown in Fig. 4.5 (B).

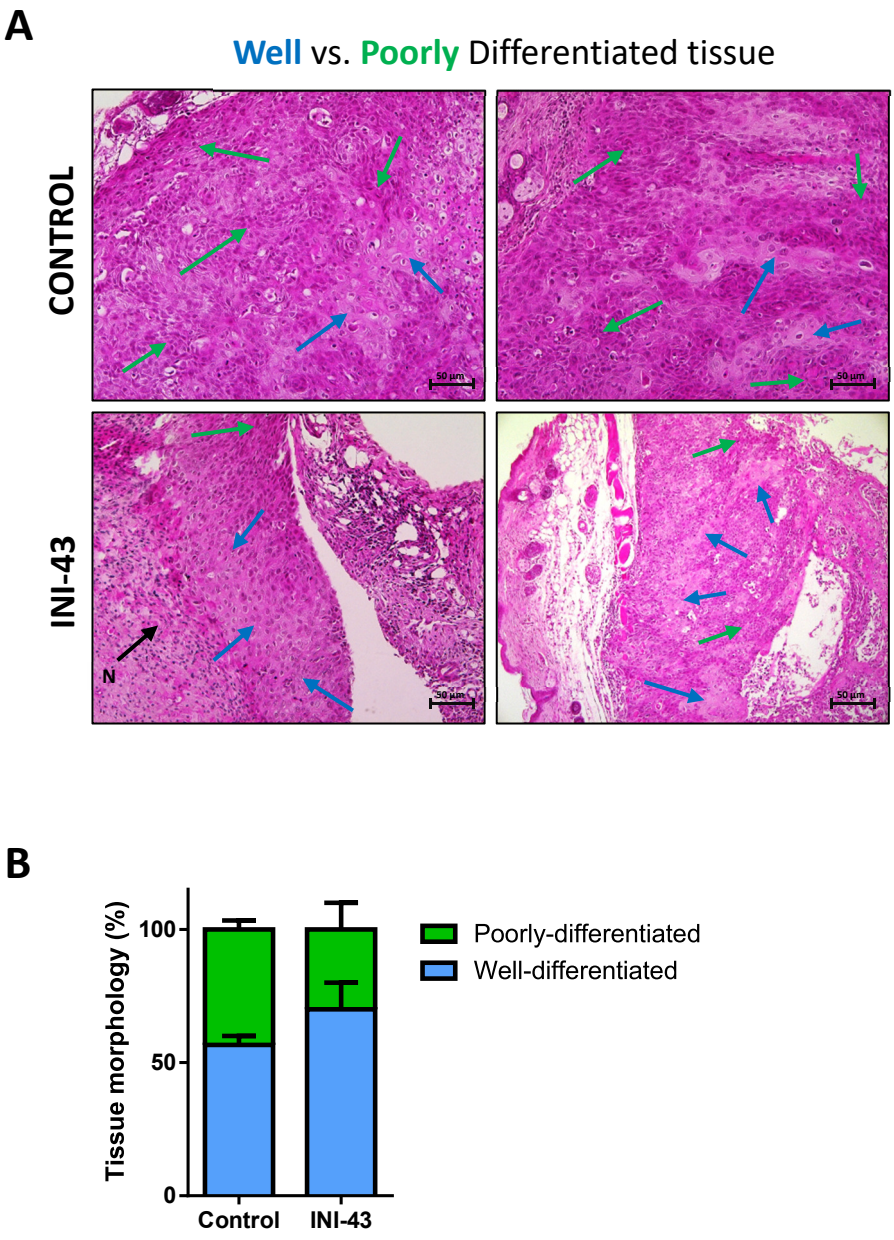


Figure 4.5: Tumour morphology following INI-43 treatment. **A)** H&E stained histological sections showing poorly-differentiated (green arrow) and well-differentiated (blue arrow) tissue in control (DMSO) and INI-43-treated tumours. (N) Region of necrosis. Scale bar= 50 µm, magnification x200. **B)** Bar graph showing the average percentage of tissue that was poorly or well-differentiated across the 3 control and INI-43-treated tumours shown as the mean ± SEM.

4.2.4.2 The effect of INI-43 on other histological features relevant to cancer

The role of necrosis, cystic change and inflammatory stroma may also be relevant to the progression of cancer and the overall outcome of patients. Staining with H&E allowed the detection of these histological features within the xenograft tumour samples. Our main observations are summarised in Table 4.2 and include; 1) that extensive necrosis formed part of all the tumours regardless of treatment, 2) the presence of cystic change was substantially less in INI-43-treated tumours in comparison to control untreated tumours and 3) the presence of stroma containing inflammatory cells was virtually absent in INI-43-treated tumours but easily identifiable untreated tumours. Representative images of the presence of these histological features (necrosis, cystic change and inflammation) are presented in Fig. 4.6 (A). Also noted was that while infiltrating, inflammatory stroma can be seen surrounding cancer tissue in the control tumours, it is minimally detected in INI-43-treated tumours (Fig. 4.6 B). The effect of nuclear import inhibition on cystic change and inflammation within the tumour may contribute to its anticancer effect.

TABLE 4.2: Histological analysis of xenograft tumour samples

CASKI	DMSO			INI-43		
	1	2	3	1	2	3
Necrosis	+++	+++	+++	+++	+++	+++
Cystic change	-	P	P	-	-	-
Inflammatory stroma	++	+	+	-	-	+

***Note: - = Absent, + = Minimal, ++ = Average, +++ = Extensive, P= Present

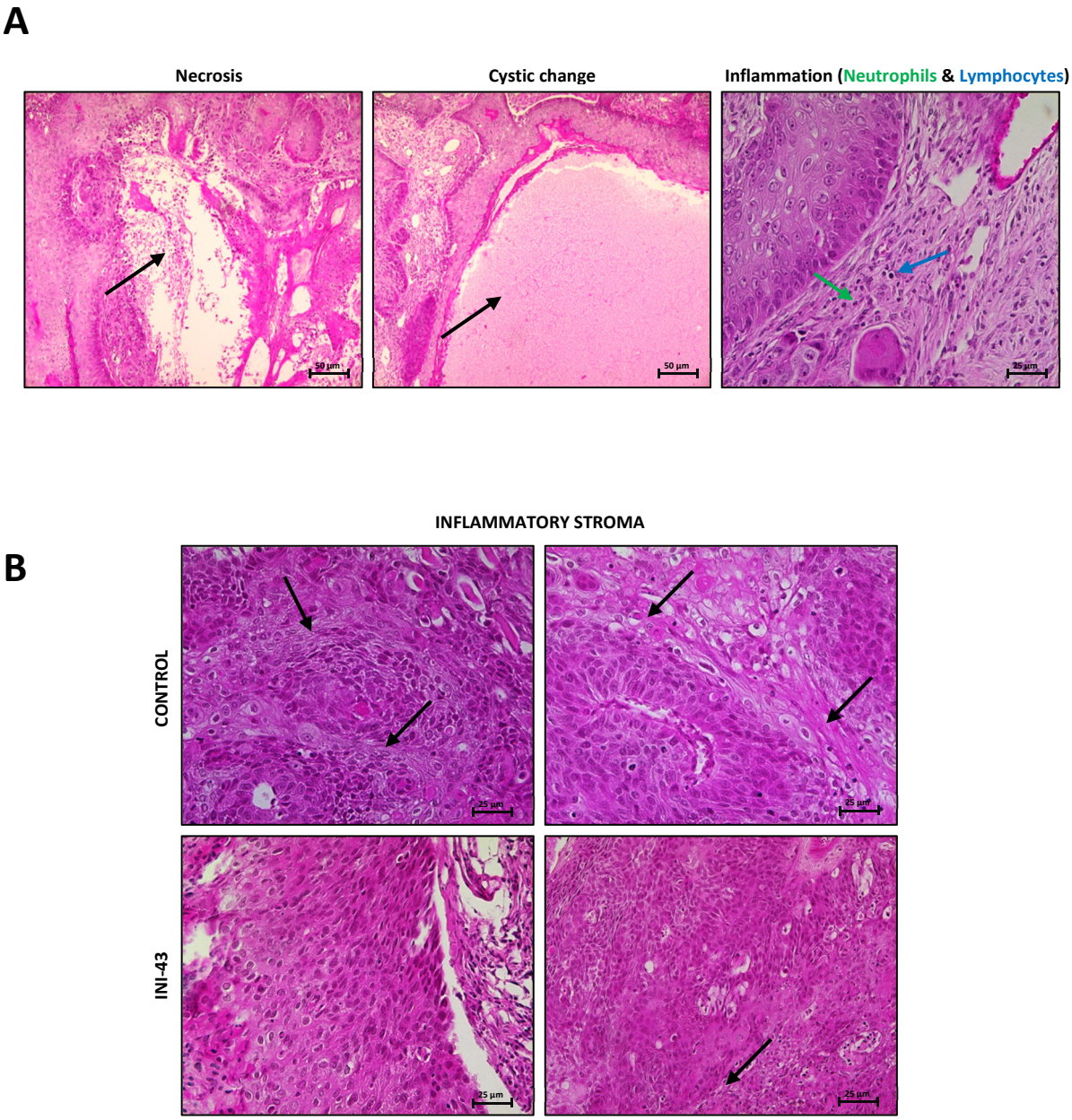


Figure 4.6: Representative images of histological findings. A) Representative H&E stained tumour sections with arrows indicating necrosis, cystic change (Scale bar= 50 µm, magnification x200) and inflammation containing neutrophils (green) and lymphocytes (blue) (Scale bar= 25 µm, magnification x400). **B)** H&E stained sections showing the presence (shown by arrow) or absence of inflammatory stroma in control and INI-43-treated tumours. Scale bar= 25 µm, magnification x400.

4.2.5 The effect of INI-43 on the expression of ECM components

4.2.5.1 Effect of INI-43 on Type I and Type IV collagen expression

The histological data presented above showed that INI-43-treated tumours contained minimal stroma surrounding cancer cells. Although stroma is predominantly a product of host fibroblasts, cancer cells can also produce ECM components thereby contributing to the generation of their own niche [238]. Collagen is a predominant component of stroma and the ECM. The observation of diminished stroma in the INI-43-treated tumour environment prompted us to investigate the effects of INI-43 on collagen expression in cancer cells. Type I collagen forms the predominant component of the interstitial matrix while Type IV collagen is the main component of basement membranes, and both have been implicated in cancer cell proliferation and motility [238, 239]. We therefore investigated the effects of INI-43 on Type I and Type IV collagen expression in CaSki, cervical cancer cells. Results show that CaSki cells treated with INI-43 showed no significant change in Type I alpha I collagen (COL1A1) expression in the presence or absence of PMA stimulation (Fig. 4.7 A & B). INI-43 treatment however, significantly reduced the expression of Type IV alpha I collagen (COL4A1) expression in both unstimulated (Fig. 4.7 C) and PMA stimulated cells (Fig. 4.7 D). This data suggests that INI-43 treatment may have an effect on the levels of ECM components.

4.2.5.2 Effect of INI-43 on the ECM degrading enzyme, MMP-9

The expression of Type IV collagen degrading enzymes such as MMP-9 have also been found to be upregulated in cancer and contribute to poor cellular differentiation and increased invasion of cancer cells [244]. We were therefore interested in monitoring the effect of INI-43 on PMA-stimulated MMP-9 expression in CaSki cells. Our results showed an increase in MMP-

9 expression with PMA stimulation which was significantly inhibited by pre-treatment with INI-43 (Fig. 4.7 E).

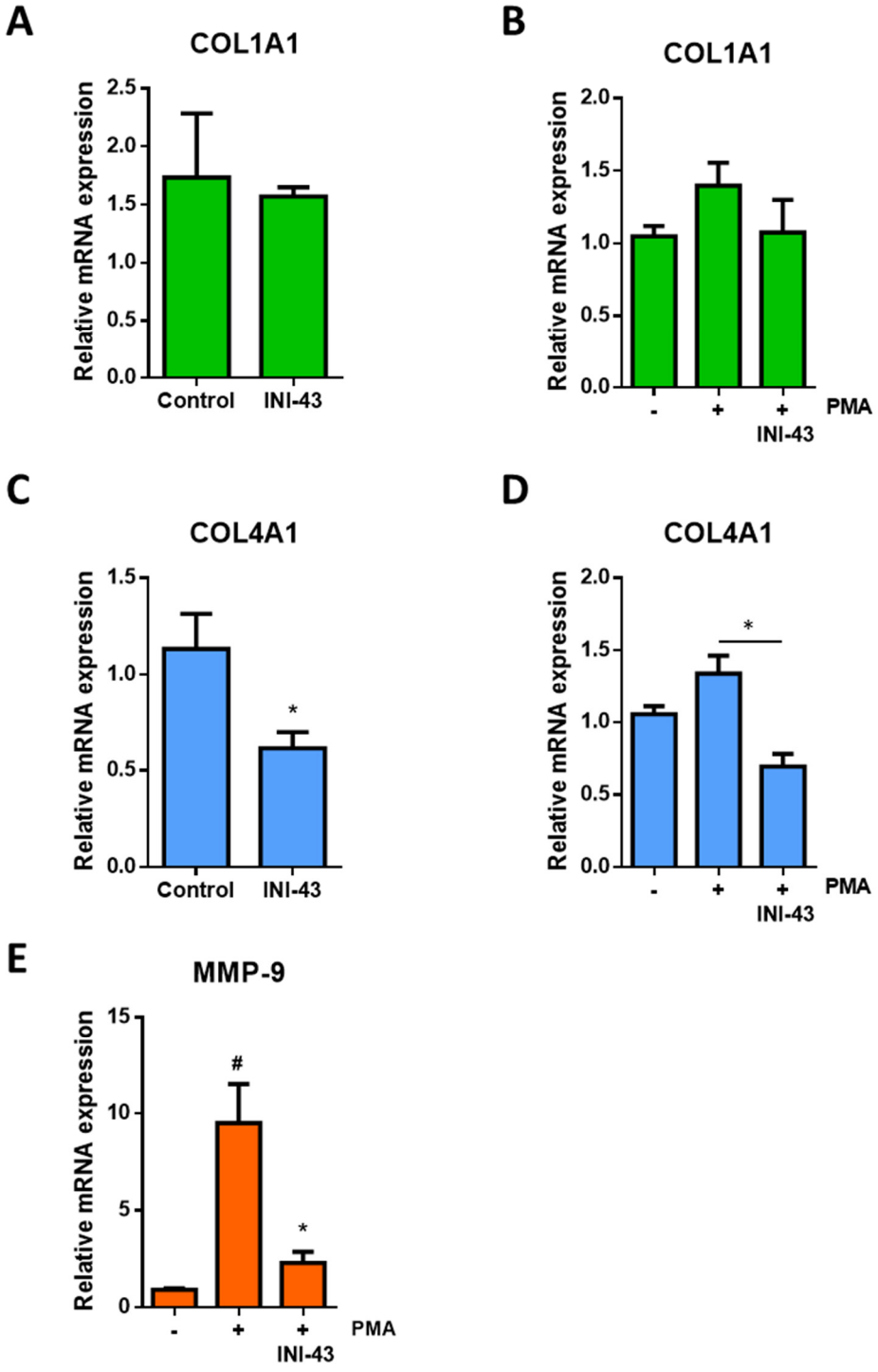


Figure 4.7: The effect of INI-43 on the expression of ECM components *in vitro.* A) qRT-PCR was used to measure mRNA expression of collagen type I (COL1A1) following a 24 hr treatment with 5 µM INI-43 alone or **B)** including a 3 hr stimulation with 0.5 µM PMA. **C)** mRNA expression of collagen type IV (COL4A1) following a 24 hr treatment with 5 µM INI-43 alone or **D)** including a 3 hr stimulation with 0.5 µM PMA. **E)** mRNA expression of MMP-9 following a 24 hr treatment with 5 µM INI-43 including a 3 hr stimulation with 0.5 µM PMA. Results shown are the mean ± SEM of experiments performed in triplicate and repeated three independent times. ($^{*}p< 0.05$).

Together these results suggest that the inhibition of nuclear import associated with KPNB1, using the small molecule inhibitor: INI-43, results in modification of the expression of ECM components and ECM modulating factors.

4.2.6 Effect of INI-43 on cancer cell morphology

Having observed a reduction in poorly-differentiated, invasive and proliferative tissue in *in vivo* tumours treated with INI-43, we postulated that INI-43 treatment may cause changes in cancer cell morphology. Cancer cells undergo a shift in morphology from being more epithelial in nature to becoming more mesenchymal which facilitates motility and promotes their invasiveness.

4.2.6.1 The effect of INI-43 on β-catenin

The protein, β-catenin, which forms part of adherens junctions plays an important role in morphogenic change of epithelial cells. In cancer, it causes the cells to lose their tight cell-to-cell adhesion and assume a more mesenchymal phenotype by releasing from the cell membrane and translocating to the nucleus. In the nucleus β-catenin exerts its effects through regulating genes that promote motility and inhibit adhesion [245]. We investigated the effect of INI-43 on β-catenin and found that INI-43 treatment of CaSki cells caused a significant reduction in the nuclear presence of β-catenin (Fig. 4.8 A). A scan of immunofluorescent intensity across the cell diameter shows localisation of β-catenin (Cy-3) overlaps with the DAPI stain (nucleus) in control cells and a reduction in β-catenin fluorescent intensity in the nucleus of INI-43-treated cells (Fig. 4.8 B). Quantification of β-catenin nuclear intensity data shows a significant reduction following INI-43 treatment (Fig. 4.8 C). The overall expression of β-

catenin remained unchanged as observed by quantification of the whole cell fluorescent intensity (Fig. 4.8 D).

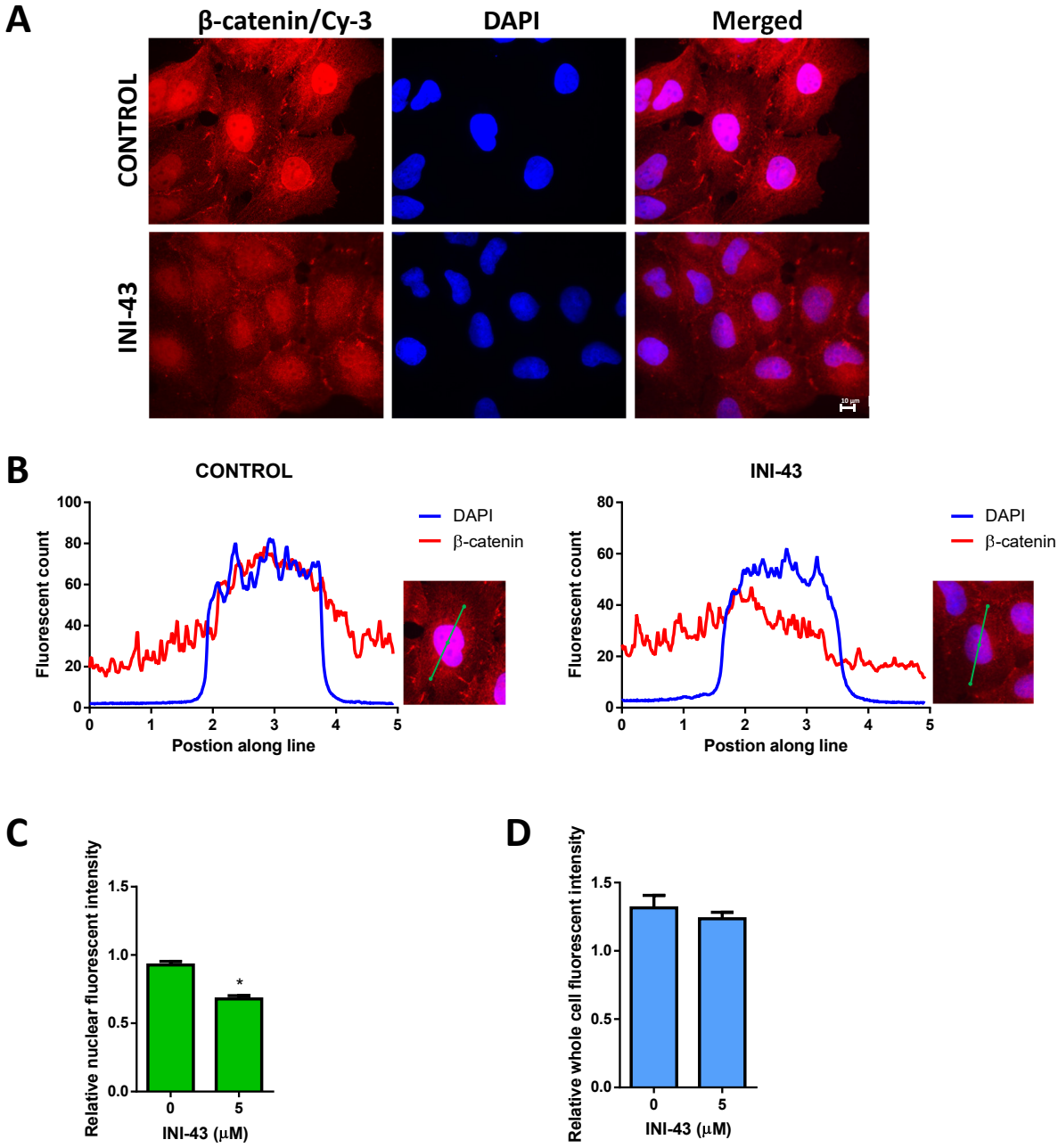


Figure 4.8: Expression and localisation of the mesenchymal marker, β-catenin, following INI-43 treatment *in vitro*. A) Representative immunofluorescent images showing β-catenin (Cy3-red) expression and localisation in CaSki cells following 24 hr treatment with 5 µM INI-43. DAPI (blue) was used to stain nuclei. Scale bar= 10µm, magnification x1000. **B)** Correlation between Cy3 (β-catenin) and DAPI (nucleus) fluorescent intensity in control and INI-43-treated CaSki cells performed using ImageJ. **C)** Quantification of β-catenin localisation across 50 cells per treatment showing the relative nuclear fluorescent intensity. **D)** Quantification of the mean whole cell fluorescent intensity per treatment. Results shown are the mean ± SEM of experiments performed two independent times. ($^{*}p < 0.05$).

4.2.6.2 Effect of INI-43 on cytoskeletal rearrangement

A reduction in nuclear β-catenin has been reported to correlate with morphological changes such as a loss in mesenchymal-like characteristics and a gain in epithelial characteristics. Thus, to further investigate the effects of INI-43 on the morphology of cancer cells, CaSki cells were stained with Phalloidin which binds polymeric filamentous actin (F-actin) and allows visualisation of the cytoskeletal arrangement. Fluorescent images showed that cells treated with INI-43 appeared to be smaller in size, more closely packed and had considerably fewer cytoplasmic protrusions (Fig. 4.9 A). To quantify this data, 50 cells per condition were monitored. The number of cytoplasmic protrusions per cell were counted and results show that INI-43 treatment significantly reduced the number of cytoplasmic protrusions (Fig. 4.9 B). In addition, a small but significant reduction in cell size was observed (Fig. 4.9 C). A reduction in cell size or the number of cytoplasmic protrusions is consistent with the development of more epithelial characteristics suggesting that INI-43 treatment contributes to a change in cancer cell morphology towards that of a more epithelial morphology.

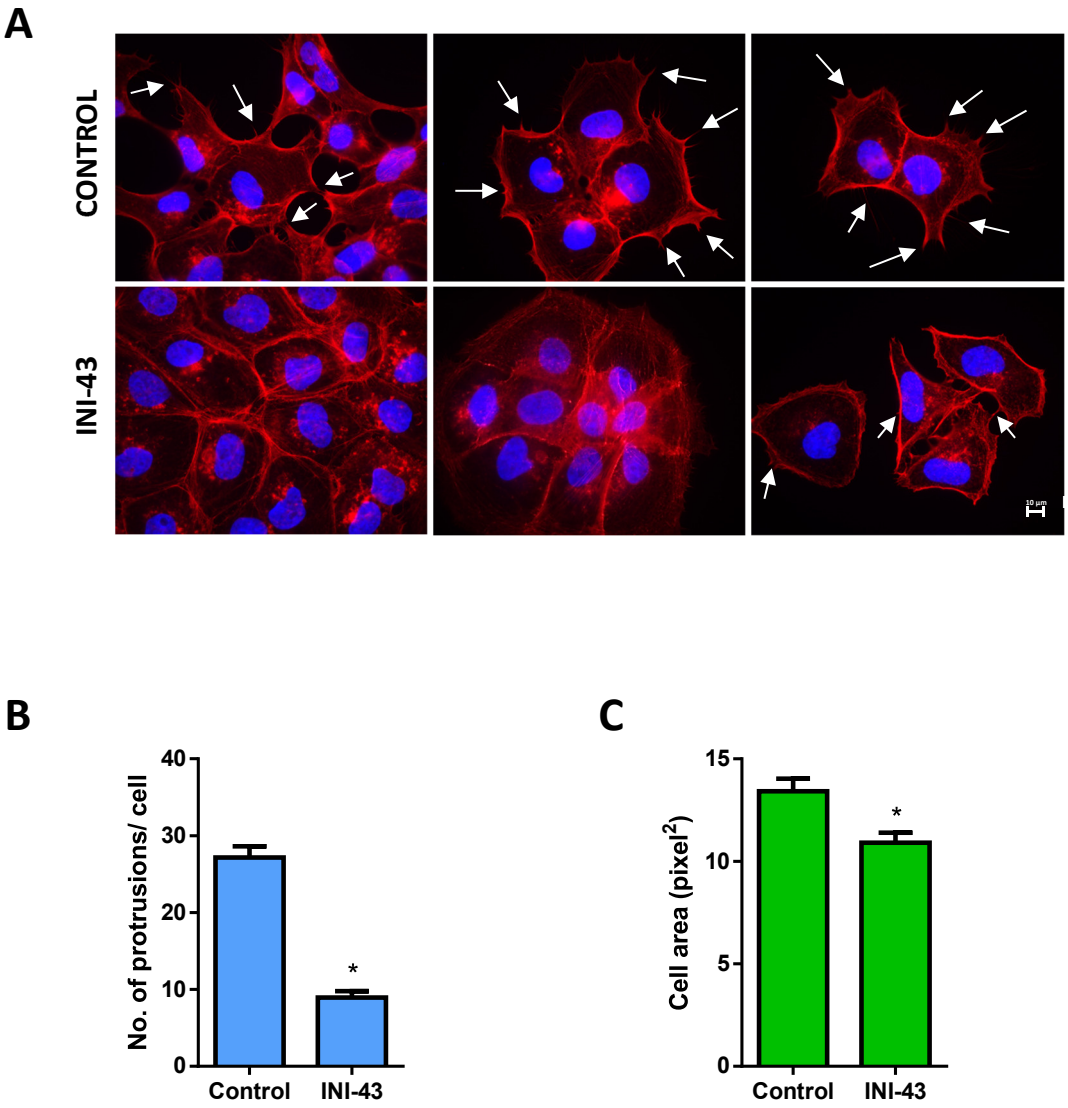


Figure 4.9: Analysis of F-actin rearrangement following INI-43 treatment *in vitro*. A) Representative immunofluorescent images of CaSki cells stained with Phalloidin (red) for F-actin and DAPI (blue) for the nuclei following treatment with 5 µM INI-43 for 24 hrs. Arrows indicate cytoplasmic protrusions. Scale bar= 10µm, magnification x1000. **B)** Bar graph representing the number of cytoplasmic protrusions per cell following INI-43 treatment. **C)** The area of 50 cells per treatment was measured using ImageJ and represented using a bar graph. Results shown are the mean ± SEM of experiments performed two independent times. ($^{*}p < 0.05$).

4.3 DISCUSSION

In this study, we investigated the pre-clinical potential of INI-43 as an anticancer drug, following initial *in vitro* studies. For this, we used an *in vivo* mouse model. CaSki, cervical cancer, cells formed ectopic xenograft tumours in nude mice. Prior toxicology studies determined that the mice used in our study tolerated a dose of 50 mg/kg INI-43 with no side effects observed. This dose was thus used to investigate the effects of INI-43 on *in vivo* tumour development. Treatment with 50 mg/kg INI-43 three times per week significantly reduced tumour size *in vivo.* This finding is significant and to our knowledge is a first to test and show the anticancer ability of a potential nuclear import inhibitor *in vivo*. There is however substantial data in the literature for the use of nuclear export inhibitors as anticancer drugs in mouse models. The Selective Inhibitors of Nuclear Export or SINE compounds, KPT-330 and KPT-251 that target the nuclear exporter XPO1, have been shown to significantly reduce tumour growth of a renal cell carcinoma xenograft mouse model [217, 246]. KPT-330 was also able to reduce tumour growth in a pancreatic orthotopic and subcutaneous mouse model [214]. Similar to the reported effects seen in these studies our study showed that tumour growth could be inhibited, exhibiting a cytostatic effect. Selinexor, KPT-330, is currently being used in a wide selection of clinical trials against solid tumours and haematological malignancies [192]. In addition, inhibition of XPO1 has been found to sensitise cells to conventional chemotherapeutic drugs and inhibitors of nuclear export have shown potential in combination therapies *in vitro* [197]. Similarly, inhibition of KPNB1 using INI-43 sensitised cervical cancer cells in culture to Cisplatin treatment (unpublished data; A. Chi, PhD Thesis, 2016[‡]). This suggests that INI-43 also has the clinical potential to be explored further in combination therapies.

[‡]A. Chi, "Investigating a novel small molecule inhibitor of nuclear import as an anti-cancer approach." PhD Thesis, University of Cape Town, 2017.

As INI-43 is suggested to physically target and inhibit KPNB1 function, we investigated whether this had any effect on the expression or localisation of KPNB1 within INI-43-treated tumours. We found that with INI-43 treatment, more cells showed cytoplasmic KPNB1 staining compared to nuclear staining in comparison to the untreated control tumours. The KPNB1 staining pattern also appeared reduced in the cytoplasm. These results are consistent with *in vitro* data from our laboratory that showed that INI-43 not only caused the cytoplasmic retention of KPNB1 but also caused a reduction in cytoplasmic and nuclear levels as seen in experiments using immunofluorescence and cyclohexamide treatment to inhibit protein synthesis [203]. The reduction in KPNB1 levels is attributed to an increase in its degradation in response to INI-43 treatment (unpublished data; S. Carden, MSc Dissertation, 2017[§]).

KPNB1 was found to be highly expressed in the cytoplasmic and nuclear compartments of diffuse large B-cell lymphoma, hepatocellular carcinoma and gastric cancer tissue. In all cases this correlated with increased Ki-67 expression, a marker of proliferation, and shorter overall patient survival [142, 247, 248]. In hepatocellular carcinoma and gastric cancer, both solid tumours, KPNB1 overexpression correlated with poorly-differentiated tumours as well as Ki-67 staining [142, 248]. Our data is in support of this as control tumours showed higher KPNB1 expression, a greater percentage of poorly-differentiated tissue and increased Ki-67 staining. In contrast the INI-43-treated tumours showed reduced KPNB1 expression, a greater percentage of well-differentiated tissue and reduced Ki-67 staining. The reduced proliferation and presence of better differentiated tissue in tumours where KPNB1 expression was reduced suggests that KPNB1 plays a role in cervical cancer tumour development. Extensive research has been done describing KPNA2, one of KPNB1's adaptor proteins, as a prognostic marker in

[§] S.Carden, "Modulating the expression and activity of the nuclear import protein, Karyopherin B1, in cancer cells." MSc Dissertation, University of Cape Town, 2017

cancer. The close relationship between these two proteins suggests that the clinical relevance of KPNA2 expression and localisation in tumours may be applicable to KPNB1 as well. Increased nuclear expression of KPNA2 has also been found to correlate with poor tissue differentiation and increased Ki-67 expression in hepatocellular and oesophageal squamous cell carcinoma [178, 249]. Erben *et al.* (2015) found that the cytoplasmic or nuclear expression pattern of KPNA2 affected patient outcome in head and neck squamous cell cancer. Patients with tumours expressing high levels of nuclear and cytoplasmic KPNA2 had a better prognosis than patients expressing only high levels of nuclear KPNA2 and no cytoplasmic expression [250]. This data suggests that not only expression but also cellular localisation of the nuclear import protein may be relevant to cancer progression. The association between KPNB1 expression and localisation with patient clinical outcome is of interest to our research group and forms part of on-going research in our laboratory.

On identifying other histological differences between control and INI-43-treated tumours that associate with the anticancer effect of INI-43, it was found that INI-43-treated tumours were absent of or contained far less inflammatory stroma. A possible reason for this could be the prevention of leukocyte migration into the tumour which has been suggested to have anti-inflammatory effects and prevents tumour progression [207]. The development of stroma and infiltration of immune cells into the tumour is largely dependent on the communication between cancer cells and the host microenvironment through chemokines and cytokines [44, 239, 241, 251]. Although the components of the ECM are mainly produced by the stromal cells, cancer cells too can produce their own ECM components and this is thought to contribute to the production of their own niche [252-254]. Among these ECM components are Type I collagen, a major component of interstitial matrix, and Type IV collagen, a major component of basement membranes. Studies in pancreatic cancer found that the cancer cells produced far greater quantities of Type IV collagen in comparison to Type I and that Type IV

collagen associated much more closely with cancer cells in tumours [255, 256]. Our study showed that INI-43 treatment of cervical cancer cells reduced the expression of Type IV collagen. Type IV collagen has been found to be important for adhesion, migration, survival, proliferation and differentiation of cancer cells. Inhibition of Type IV collagen using siRNA was able to significantly reduce cell growth and migration while increasing apoptosis in pancreatic cancer cells [253]. Type IV collagen was also able to induce EMT in breast cancer cells [257]. The expression of Type IV collagen is regulated by a tight balance between MMP-2 and -9 and their inhibitors, TIMP-1 and TIMP-2 [258]. Following INI-43 treatment it was found that MMP-9 expression was significantly reduced. As cancer cells adopt a more mesenchymal phenotype which favours migration and invasion they show increased MMP expression, changes in the cytoskeleton to facilitate this as well as β-catenin nuclear localisation among other features [257]. Our data shows that INI-43 treatment was able to reduce β-catenin nuclear localisation as well as cause cytoskeletal changes such as; causing a reduction in the number of cytoplasmic protrusions and cell size. These features are characteristic of a more epithelial, tightly-packed phenotype. In gastric cancer tumours, increased nuclear KPNA2 expression associated with increased nuclear accumulation of β-catenin while reduced KPNA2 expression correlated with cytoplasmic β-catenin expression [122]. This suggests that increased expression of the nuclear transporter associates with EMT even though β-catenin nuclear import is independent of the Karyopherin family [259]. KPNA2 knockdown was able to reverse EMT [139]. This evidence suggests that the reduced nuclear localisation of β-catenin following INI-43 treatment, is not necessarily directly associated with KPNB1 inhibition and that an alternative mechanism may exist.

In conclusion, this study has determined that INI-43 is able to inhibit tumour growth *in vivo.* Reduced KPNB1 expression and altered localisation may contribute to cancer progression and could explain the reduced Ki-67 expression and presence of more well-differentiated tissue in

INI-43-treated tumours. Inhibition of KPNB1 in cancer cells may also disrupt the expression of genes required for communication with the tumour microenvironment and establishment of the ECM. Together these results begin to elucidate the role of INI-43 *in vivo* and provide further evidence of its anticancer properties. Further investigation into how KPNB1 inhibition affects the communication between cancer cells and other stromal cells would be of interest.

CHAPTER 5

CONCLUSION

Targeted therapy is a novel and innovative chemotherapeutic approach which identifies a molecular lesion or dysregulated pathway that cancer cells are dependent on. Key components of the nuclear transport system including the nuclear import proteins; KPNB1 and KPNA2, and nuclear export protein, XPO1, have been identified in our laboratory and others as potential therapeutic targets and prognostic markers [122, 128, 130, 138, 142, 169, 175, 260]. This study set out to investigate the role of KPNB1 in cancer cell biology changes and inflammatory signalling associated with tumourigenesis. The nuclear import protein, KPNB1, was inhibited using targeted siRNA and a small molecule inhibitor, INI-43, and changes in cancer cell proliferation, survival, migration and invasion monitored *in vitro*. To elucidate the mechanism behind these cell biology changes the effect of KPNB1 inhibition of transcription factors associated with cancer cell biology such as NFkB and AP-1 and inflammatory target gene expression were investigated. The pre-clinical anticancer potential of INI-43 treatment was also further investigated using an ectopic xenograft mouse model. Our study aimed to further characterise the anticancer potential of KPNB1 inhibition.

Previous studies in our laboratory using cervical tissue, found KPNB1 to be overexpressed in cervical cancer tissue compared to normal cervical tissue suggesting it may have value as a chemotherapeutic target [128]. In this study and others, KPNB1 was found to be essential for cancer cell proliferation and survival while normal cell proliferation and survival was relatively unaffected by the inhibition of KPNB1 [202, 203, 247, 248]. These findings indicated that cancer cells have a greater dependency on KPNB1 than normal cells for their proliferation.

Little however, is known about the role of KPNB1 in other cancer cell biological phenotypes such as migration and invasion. In this study, we show that inhibition of KPNB1 using either siRNA or the small molecule, INI-43, significantly reduced the migration and invasion of cervical cancer cells. Treatment with INI-43 was also found to negatively regulate matrix metalloproteases (MMPs), which facilitate cell motility, and was positively associated with elevated levels of endogenous inhibitors of MMPs. Our results showed that INI-43 treatment reduced PMA-stimulated MMP-2 and -9 expression and increased TIMP-1 and -2 expression. Both MMP's and TIMPs are tightly regulated by inflammatory transcription factor signalling and their dysregulation has been associated with the progression of cancer [40, 88, 104]. Considering this we proposed that KPNB1 may be the key transporter of transcription factors associated with cancer cell growth, migration and invasion.

Two such transcription factors closely involved in the development and maintenance of the hallmarks of cancer are, NFkB and AP-1 [74, 88]. Nuclear import of these transcription factors is essential for their activity. We thus questioned a potential role of KPNB1 in the nuclear import of these transcription factors. This was achieved by inhibiting KPNB1 and monitoring effects on NFkB and AP-1. Using western blotting and immunofluorescent microscopy, we showed that KPNB1 is essential for the nuclear import of NFkB. Transcriptional activity assays, using luciferase reporter assays for NFkB and AP-1, showed that the activities of both transcription factors were negatively impacted when KPNB1 was inhibited using KPNB1 siRNA and INI-43. The inhibitory effect on transcriptional activity was comparable to that of commercially available nuclear import inhibitors; Importazole and Ivermectin. Interestingly, a lower concentration of INI-43 (5 μM) has the same inhibitory effect as Importazole and Ivermectin used at concentrations greater than 10 μM, suggesting that INI-43 is a more potent inhibitor.

Next we investigated whether the effect of KPNB1 inhibition on NFkB and AP-1 transcriptional activity translated to the level of target gene expression. The expression of key inflammatory genes which are known targets of NFkB and/or AP-1; IL-1β, IL-6, TNF-α and GM-CSF was monitored in cervical cancer cells in response to KPNB1 inhibition. These inflammatory cytokines have been implicated in promoting proliferation and facilitating cancer cell migration and invasion [46, 231-233]. Inhibition of KPNB1 significantly inhibited PMA-stimulated expression of these cytokines in cancer cells. The substantially greater reduction in IL-1β and TNF-α expression following INI-43 treatment compared to the effect of the NFkB inhibitor, JSH-23, and in IL-6 expression compared to the JNK inhibitor, SP600125, suggests that KPNB1 inhibition may have an effect on multiple transcription factors. The activity and expression of the IL-6 target gene was investigated further as this gene is under the control of both the NFkB and AP-1 transcription factors. Interleukin 6 promoter activity was significantly reduced following KPNB1 inhibition using both siRNA and INI-43. INI-43 treatment also significantly reduced the IL-6 protein secretion from HeLa and SiHa cells. Our data also showed that while INI-43 caused a significant reduction in IL-6 mRNA expression in cervical cancer cell lines; HeLa, SiHa and CaSki, little/no effect was detected on normal fibroblast IL-6 expression at the same concentration. These results support our earlier findings that cancer cells are more sensitive to the effects of KPNB1 inhibition. Cancer cells are known to become "addicted" to certain transcription factors or oncogenes and could be the reason why cancer cells are more reliant on KPNB1 than normal cells [236].

To further elucidate whether either NFkB or AP-1 are required for the migration of cervical cancer cells, each transcription factor was individually inhibited using JSH-23 and SP600125 alongside INI-43 and the effect on migration analysed. Inhibition of the AP-1 signalling pathway using SP600125 significantly reduced migration and inhibition of NFkB using JSH-23 showed a downward trend towards inhibiting migration that was however, not significant.

Neither inhibition of AP-1 or NFkB on their own reduced the migratory potential of cancer cells to the extent of that observed with INI-43. This further suggests that the effects seen following KPNB1 inhibition are mediated through more than one transcription factor signalling pathway. It is not uncommon for transcription factors to act synergistically to achieve enhanced transcriptional regulation of the same target gene. Transcription factors of the JUN/FOS, CREB/ATF and NFkB families most commonly act together to enhance expression of a single target gene [234]. Darnell (2002) proposed that targeting nuclear import proteins may be an effective way of inhibiting multiple overactive signalling pathways [23]. Our findings are in support of this proposal, showing that KPNB1 inhibition affects the transcriptional activity of both NFkB and AP-1.

Extensive *in vitro* studies using cervical cancer cell lines in tissue culture to provide evidence for the role of KPNB1 as an anticancer target has been performed however, little is known about the *in vivo* effects of KPNB1 inhibition on tumour development. A significant finding from our study showed that INI-43 treatment inhibited tumour growth in an ectopic xenograft mouse model. We had previously reported that the cellular and signalling effects of KPNB1 inhibition using either siRNA or INI-43 yielded very comparable results, supporting that INI-43-induced anticancer activity is mediated through KPNB1. We therefore investigated whether INI-43 treatment of tumours influenced the expression or localisation of KPNB1. Our study found that INI-43 treatment resulted in more tumour cells showing cytoplasmic KPNB1 staining compared to nuclear staining in comparison to the DMSO-treated control tumours. The KPNB1 staining pattern also appeared reduced in the cytoplasm. These results were in support of our *in vitro* data in our laboratory that showed that INI-43 not only caused the cytoplasmic retention of KPNB1 but also caused a reduction in cytoplasmic and nuclear levels as seen in experiments using immunofluorescence and cyclohexamide treatment to inhibit protein synthesis [203]. The reduction in KPNB1 levels was attributed to an increase in its

degradation in response to INI-43 treatment (unpublished data; S. Carden, MSc Dissertation, 2017[**]). The cellular localisation and expression levels of KPNB1 and its adaptor protein KPNA2 have been correlated to proliferation and differentiation status of the tumour as well as patient outcome [142, 178, 179, 248, 249]. Increased KPNB1 expression positively correlated with tumour grade and size and associated with poor survival in patients with hepatocellular and gastric carcinoma [142, 248]. Patients with tumours expressing high levels of nuclear and cytoplasmic KPNA2 had a better prognosis than patients expressing only high levels of nuclear KPNA2 and no cytoplasmic expression [250]. In support of these findings our data shows that INI-43-treated tumours, expressing lower levels of cytoplasmic KPNB1, had reduced levels of proliferation (Ki-67 staining) and appeared more well-differentiated. In comparison, the control tumours, expressing higher levels of KPNB1, had increased levels of proliferation and appeared poorly-differentiated. These results implicate KPNB1 as being required for cervical cancer tumour progression.

On further investigation of other histological differences between control and INI-43-treated tumours, that associate with the anticancer effect of INI-43, it was found that INI-43-treated tumours were absent of or contained far less inflammatory stroma. Our previous findings show that KPNB1 inhibition reduced NFkB and AP-1 activity consequently leading to the reduced expression of inflammatory cytokines. These inflammatory cytokines play an important role in the recruitment and development of the inflammatory stromal components making up the ECM [197, 239, 241, 251]. This could provide an explanation for the absence of inflammatory stroma in the INI-43-treated tumours. Although most components of the ECM are produced by fibroblasts or other host cells, cancer cells behave aberrantly and can contribute to the production of their own niche [252, 253]. This led us to investigate the role

[**] S.Carden, "Modulating the expression and activity of the nuclear import protein, Karyopherin B1, in cancer cells." MSc Dissertation, University of Cape Town, 2017

of KPNB1 inhibition in cancer cells on the expression of certain ECM components. We found INI-43 treatment to inhibit the expression of type IV collagen in cancer cells, an ECM component important for cancer cell adhesion, proliferation and motility [253]. Another factor important for cancer cell motility, MMP-9, was also inhibited by INI-43 treatment. Together these results suggest that INI-43 treatment influences the formation of the tumour stroma, contributing to its anticancer activity.

An increase in MMP expression is also characteristic of cells of a mesenchymal phenotype [257]. The enhanced activation of NFkB in cancer cells has been shown to promote the progression of cancer cells to a more mesenchymal-like phenotype by inhibiting E-cadherin expression, a characteristic epithelial marker [47]. In more mesenchymal cells, the loss of E-cadherin from adherens junctions activates cytosolic β-catenin and stimulates its nuclear translocation [245]. A mesenchymal phenotype facilitates migration and invasion of cancer cells. We therefore investigated the effect of INI-43 treatment on changes in the cytoskeleton and β-catenin nuclear localisation both characteristic features of the transition between epithelial and mesenchymal phenotypes. Our data shows that INI-43 treatment could reduce β-catenin nuclear localisation as well as cause cytoskeletal changes including; a reduction in the number of cytoplasmic protrusions and a reduction in cell size. Together these findings suggest that INI-43 treatment is causing a loss of mesenchymal-like features of the cancer cells assayed in our study, likely to affect their invasive capabilities.

The key findings of this study are summarised diagrammatically in Figure 5.1. During normal cell growth conditions, KPNB1-mediated nuclear import cargo proteins such as transcription factors containing a NLS are transported into the nucleus together with or without the Karyopherin alpha adaptor protein. Once in the nucleus the transcription factor is available to bind the DNA and induce target gene expression (Fig. 5.1 A). In various cancers KPNB1 has

been shown to be overexpressed [128]. This overexpression has been associated with enhanced nuclear import rates of KPNB1 cargoes including transcription factors such as NFkB and AP-1 [133, 247]. Once in the nucleus the increased activity of these transcription factors associates with the increased proliferation, survival, increased motility and inflammation seen in cancer (Fig. 5.1 B). Upon treatment with the nuclear import inhibitor, INI-43, or through inhibition of KPNB1 using siRNA, NFkB and AP-1 are inhibited from entering the nucleus. The consequences of inhibiting NFkB and AP-1 nuclear localisation and activity have anticancer effects. These include; a reduction in cancer cell proliferation and increase in apoptosis. A reduction in migration and invasion of cancer cells characterised by a transcriptional switch, blocking MMP-2 and -9 expression and increasing TIMP-1 and -2 expression. Reduced expression of inflammatory cytokines; IL-6, IL-1β, TNF-α and GM-CSF, and a significant reduction in tumour growth in a cervical cancer mouse model (Fig. 5.1 C). These transcriptional changes have implications for cancer cell biology, hence the observed changes in cancer cell proliferation, migration, invasion and tumour formation when KPNB1 is inhibited.

This study provides significant evidence supporting the use of targeted therapies toward KPNB1 as an anticancer approach. Further development of KPNB1-mediated nuclear import inhibitors such as INI-43 may yield a class of clinically relevant targeted chemotherapies.

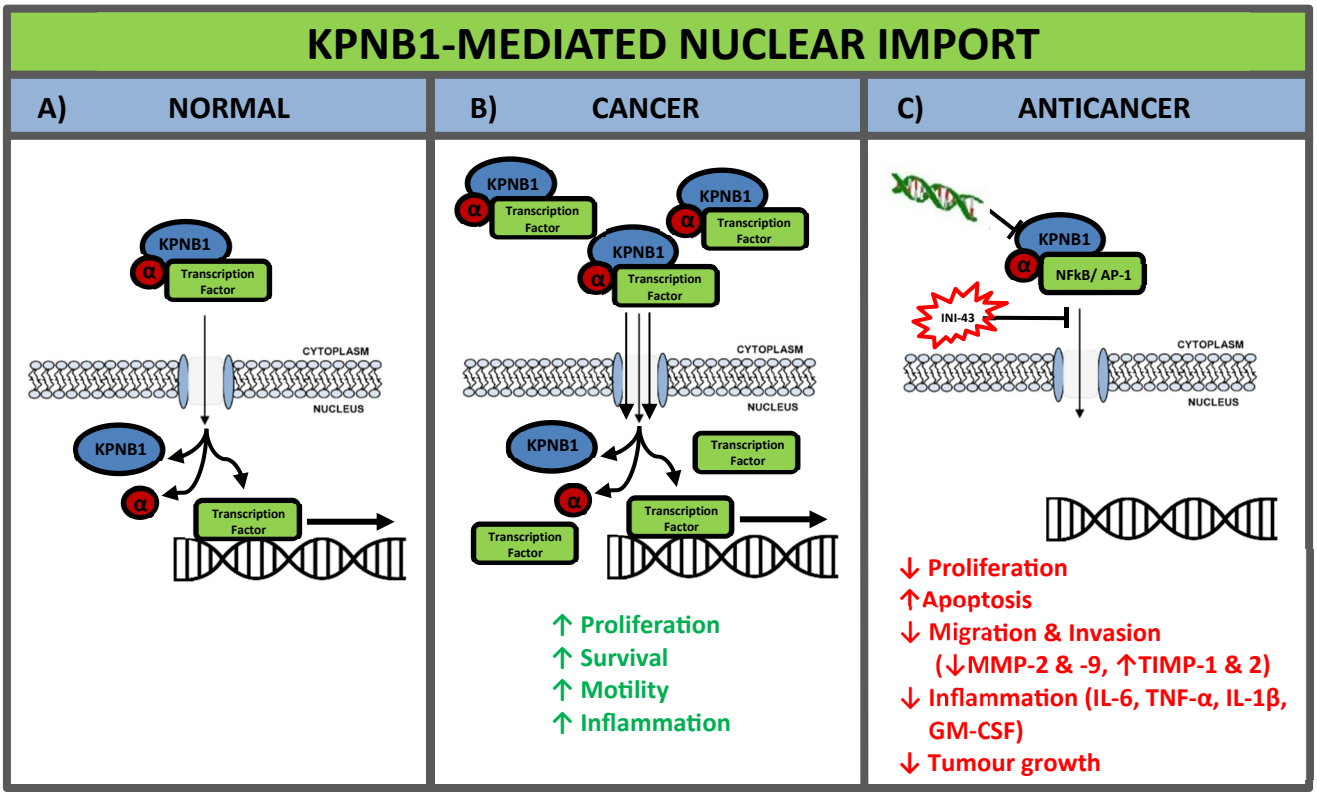


Figure 5.1: Summary of findings following KPNB1-mediated nuclear import inhibition in cancer.
A) KPNB1-mediated nuclear import in normal cells showing the transport of a transcription factor across the nuclear envelope into the nucleus using the Karyopherin alpha adapter protein (α) and KPNB1. **B)** A depiction of KPNB1-mediated nuclear import in cancer. The overexpression of KPNB1 in cancer cells allows for enhanced nuclear import rates of transcription factors responsible for increasing proliferation, survival, motility and inflammation in cancer. **C)** Inhibition of KPNB1-mediated nuclear import using siRNA or INI-43, blocked nuclear import of transcription factors; NFkB and AP-1 in cancer cells. As a result reduced proliferation, increased apoptosis, reduced migration and invasion, reduced inflammation and reduced tumour growth was observed in a cancer model.

CHAPTER 6

MATERIALS AND METHODS

6.1 MATERIALS

6.1.1 Cell lines

Human cervical carcinoma cell lines, HeLa, SiHa and CaSki were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). Normal human skin fibroblast cell culture, FGo, was obtained from the Department of Medicine, UCT.

6.1.2 siRNA

Small-interfering RNA (siRNA) was used to inhibit gene expression. The KPNB1 siRNA (sc-35736) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A non-silencing control siRNA (MISSION® Positive Control siRNA) was purchased from Sigma-Aldrich (St Louis, MO, USA). Both products were purchased as a lyophilized powder and resuspended in RNase-free water to a concentration of 10 µM and stored at -20°C.

6.1.3 Compounds

6.1.3.1 Inhibitor of Nuclear Import-43 (INI-43)

Inhibitor of Nuclear Import-43 (Chemical name: *3-(1H-benzimidazol-2-yl)-1-(3-dimethylaminopropyl)pyrrolo[5,4-b]quinoxalin-2-amine*)) was purchased from MolPort

Chemicals (MolPort-000-492-602, Riga, Latvia) as a powder. The compound was dissolved in DMSO to a stock solution of 10 mM and stored at -20°C.

6.1.3.2 Phorbol-12-myristate-13-acetate (PMA)

The phorbol ester, PMA, was purchased from Sigma-Aldrich (St Louis, MO, USA) as a powder and dissolved in DMSO to a stock concentration of 1 mM and stored at -80°C.

6.1.3.3 JSH-23

The NFkB inhibitor, JSH-23, was purchased from Sigma-Aldrich (St Louis, MO, USA) as a powder and dissolved in DMSO to a stock concentration of 25 mM and stored at -80°C.

6.1.3.4 SP600125

The selective inhibitor of c-Jun N-terminal kinase (JNK), SP600125, was purchased from Sigma-Aldrich (St Louis, MO, USA) as a powder and dissolved in DMSO to a stock concentration of 25 mM and stored at -80°C.

6.1.3.5 Ivermectin

Nuclear import inhibitor, Ivermectin, was purchased from Sigma-Aldrich (St Louis, MO, USA) as a powder and dissolved in DMSO to a stock concentration of 50 mM and stored at -20°C.

6.1.3.6 Importazole

Nuclear import inhibitor, Importazole, was purchased from Sigma-Aldrich (St Louis, MO, USA) as a powder and dissolved in DMSO to a stock concentration of 50 mM and stored at 4°C.

6.1.4 Plasmids

The NFkB p65 reporter construct in a pGL4 vector contains five copies of the p65 binding site that drives transcription of the luciferase reporter gene *luc2P* was purchased from Promega (Madison, WI, USA). The AP-1 reporter plasmid containing four AP-1-Luc binding sites was received from Professor M. Birrer (Harvard Medical School, MA, USA). This plasmid contained four wildtype AP-1 binding sites (TGAC/GTCA) upstream of the minimal promoter sequence from the albumin gene and the firefly luciferase reporter gene [261]. The full-length IL-6 promoter construct inserted into the *Bam*H1-*Hind*III sites of the pXP2-luciferase vector was kindly received from Prof L. Zerbini (ICGEB, Cape Town, South Africa) [262]. A plasmid encoding the Renilla luciferase reporter gene, pRL-TK (Promega, Madison, WI, USA), was used as an internal control for the transfection efficiency of promoter assays.

6.1.5 Antibodies

The following antibodies were used for Western Blotting, Immunofluorescent and Immunohistochemistry experiments and were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA): anti-NFkB p65 (sc-7151X), anti-NFkB p50 (sc-7178X), anti-KPNB1 (sc-11367), anti-p-c-JUN (sc-16312-R), anti-c-JUN (sc-44), anti-β-tubulin (sc-9104), anti-GAPDH (sc-47724) and anti-TBP (sc-204). Another antibody used for Immunohistochemistry was the anti-Ki-67 (M7240, Dako, Glostrup, Denmark). Secondary antibodies used were Goat anti-Rabbit IgG (H + L)-HRP Conjugate (#1706515, Bio-Rad), Goat anti-Mouse IgG (H + L)-HRP Conjugate (#1706516, Bio-Rad), Cy-3-conjugated Goat ant-rabbit (#111-165-144, Jackson ImmunoResearch) and Dako REAL EnVision-HRP Goat ant-rabbit (K4011)) or Goat anti-mouse (K4007) (Glostrup, Denmark).

6.1.6 Animals

Animal ethics approval was obtained from the Faculty of Health Sciences Animal Ethics Committee, University of Cape Town, South Africa (reference number 012/009). The live animal experiments were performed together with Ms. Alicia Chi, a PhD student in our laboratory. Female athymic nude mice (Strain: UCT 21) 4-6 weeks old were obtained from the SPF Research Animal Facility (Health Science Faculty, University of Cape Town, South Africa) and all procedures were carried out in strict accordance with the guidelines of the Facility's Animal Ethics Committee. The mice were housed in the BSL2 Research Animal Facility with 6 mice per cage in autoclaved polysulfone cages, provided with red plastic housing for enrichment and were given access to food and water *ad libitum*. The room was maintained at constant temperature and humidity with 12 hour light/dark cycles.

6.2 METHODS

6.2.1 Cell culture

6.2.1.1 Medium

All cells used were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Life Technologies, Carlsbad, CA, USA) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 10% Fetal Calf Serum (FCS) (Gibco, Life Technologies, Carlsbad, CA, USA). All cells were cultures at 37°C in a humidified chamber with 5% CO_2.

6.2.1.2 Sub-culturing cells

Cells were grown adherently in 10 cm tissue culture dishes to about 80-90% confluency before being washed with 1x PBS and removed from the plate by trypsinisation using 3 ml trypsin-EDTA solution at 37°C. Once cells had detached, the trypsin-EDTA-cell suspension was neutralised with the addition of 3 ml fresh culture medium. The cell suspension was centrifuged at 500 x G for approximately 5 minutes to pellet cells. The supernatant was removed and the cell pellet resuspended in the required volume of culture medium. Cells were generally sub-cultured at a ratio of 1:6.

6.2.1.3 Cryopreservation and Reconstitution

For long term storage of tissue culture cells, cells were grown to 80-90% confluency and collected by trypsinisation as described above. The cell pellet was resuspended on chilled freezing medium at a concentration of about 1 x 10^6 cells per ml. Cells were transferred to cryovials and frozen at -80°C before being transferred to liquid nitrogen. To reconstitute cells, a single cryovial containing 1 ml of cell suspension was thawed rapidly at 37°C in a waterbath and added to 9 ml fresh culture medium in a 10 cm tissue culture dish.

6.2.1.4 Mycoplasma test

Cells were regularly tested for mycoplasma contamination to ensure they were mycoplasma-free. Cells were grown in penicillin and streptomycin-free culture medium for 4-5 days before being plated onto glass coverslips. The cells were then fixed and stained with Hoescht fluorescent DNA-binding stain before mounting and visualisation on a Zeiss Axiovert 200M Fluorescent Microscope (Carl Zeiss, Jena, Germany).

6.2.2 Transfection

6.2.2.1 siRNA (KPNB1)

Short-interfering RNA (siRNA) was used to inhibit KPNB1 gene expression (sc-35736, Santa Cruz Biotechnology, Santa Cruz, CA, USA). Control siRNA (sc-37007, Santa Cruz Biotechnology) consisting of a scrambled RNA sequence was used as a non-silencing control. siRNAs were resuspended in RNAse-free water and used at a stock concentration of 10 µM. Cells were plated at a density of 30 000, 120 000 or 250 000 cells in a 24-well plate, 35 mm dish or 60 mm dish respectively and allowed to settle overnight. The transfection mixture was prepared in serum-free DMEM using TransFectin Lipid Reagent (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions and incubated with the cells for 7 hours. Thereafter the medium was replaced with fresh culture medium for an additional 17-41 hours. The effect of KPNB1 knockdown was confirmed by Western Blot analysis or qRT-PCR.

6.2.2.2 Plasmid

The luciferase reporter plasmids were transfected into cervical cancer cells to measure NFkB and AP-1 transcriptional activity as well as IL-6 promoter activity. Cells were seeded at a concentration of 30 000 cells/well in a 24-well plate in 500 µl fresh culture media and allowed to adhere overnight. The transfection mix per well was prepared in 50 µl antibiotic-free and serum-free culture medium. The luciferase reporter plasmid (100 ng), Renilla luciferase reporter gene (10 ng) and GeneCellin™ Transfection Reagent (0.4 µl) (Celtic Molecular Diagnostics, South Africa) was diluted in the antibiotic-free and serum-free culture medium, vortexed briefly and allowed to stand at room temperature for 15 mins before being added

drop-wise to the cells. Culture medium was replaced after 7 hours and cells allowed a further 17-41 hours to incubate before being assayed.

6.2.3 EC_{50} determination

Cells were plated in 96-well plates at a density of 5000 cells/well in 90 µl culture medium and allowed to adhere overnight. All experiments were seeded in triplicate and included cell-free media controls. For EC_{50} determination cells were treated with 10 µl of varying concentrations of drug for 48 hrs. Following treatment 10 µl MTT reagent (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma-Aldrich, USA) was added for 4 hours. Formazan crystals were solubilised using 100 µl Solubilisation solution for 24 hours. Absorbencies were measuring at OD_{595nm} using a Biotek microplate spectrophotometer (Winooski, VT, USA). The absorbance of drug treated cells was normalised to the absorbance of drug-containing culture medium and the EC_{50} value (Effective half-maximal concentration of the drug) calculated using GraphPad Prism V5.0.

6.2.4 Cell viability assay

Cells were plated in 96-well plates at a density of 2000 cells/well in 90 µl culture medium and allowed to adhere overnight. All experiments were seeded in triplicate and included cell-free media controls. For cell viability assays, cells were cultured for various time points up to 3 days following transfection with KPNB1/control siRNA. Following treatment, 10 µl MTT (Sigma-Aldrich, USA) was added for 4 hours and crystals solubilised for 24 hours with 100 µl Solubilisation solution. Absorbencies were measured at OD_{595nm} using a BioTek microplate spectrophotometer (Winooski, VT, USA). Cell viability was normalised to cell-free controls and the results plotted as a histogram showing changes in cell viability over time.

6.2.5 Caspase 3/7 Glo cell death assay

Cells were plated in 96-well plates at a density of 4000 cells/well and treated with INI-43 for various periods of time. Caspase-3/7 activity was monitored at each time point using the Caspase-Glo 3/7 assay (Promega, Madison, WI, USA), according to the manufacturer's instructions. Luminescence was measured using the Veritas microplate luminometer (Promega) and normalized to OD_{595nm} readings of MTT experiments performed in parallel.

6.2.6 Transwell migration and invasion assays

Cells were seeded into 35 mm dishes and treated accordingly before being trypsinised, resuspended in 0.1% FCS-containing DMEM and seeded at 30 000 cells/well into 12-well Transwell migration chambers (Greiner Bio-One, Austria) or 24-well matrigel-covered Transwell invasion chambers (BD Biosciences, USA) with an 8 µm pore size. The chambers were placed into a lower chamber containing 20% FCS-containing DMEM and the cells allowed to migrate/invade through the membrane or matrigel matrix over 24 hrs. The cells that were unable to move through the membrane were removed while the remaining cells were fixed in methanol, stained with crystal violet, counted and imaged under the Zeiss Primovert phase contrast microscope (Carl Zeiss, Jena, Germany). Results were normalised to an MTT cell viability assay and western blotting confirmed KPNB1 knockdown.

6.2.7 Gelatin Zymography

Cells were seeded into 35 mm plates and treated with 10 µM INI-43 for 3 hours. The treatment was removed and serum-free media placed onto the cells to condition for 16 hrs. Conditioned

media was collected and centrifuged at 500 x G to remove any cellular debri. After which it was combined with sample buffer, equal volumes loaded into a gelatin gel and run at 125 V until the tracking dye reached the bottom. The gel was removed and placed in a renaturing solution before being placed in the developing buffer and allowed to incubate at 37°C overnight to facilitate gelatinase activity. The staining solution was used to dye the gel while the destaining solution exposed areas of gelatinase activity.

6.2.8 Analysis of mRNA expression

6.2.8.1 RNA isolation

Cells were plated at a density of 250 000 cells/ 60 mm plate and cells were transfected with siRNA (48 hrs) or treated with the relevant compounds. Cells were washed with 1x PBS following treatment and RNA harvested using Qiazol (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Chloroform was added to the samples at 0.2 ml/1 ml Qiazol, mixed and incubated at room temperature for 3 mins. Samples were centrifuged at 12000 x G for 15 mins (4°C) to allow for the separation of phases. The upper aqueous phase was transferred to a new tube and the RNA precipitated in 500 µl isopropanol /1 ml Qiazol by vortexing and incubating at room temperature for 10 mins. Samples were centrifuged for another 10mins at 12000 x G. The supernatant was discarded and the pellet washed in 1000 µl 75% ethanol/1 ml Qiazol for 5 mins at 7500 x G. Ethanol was discarded and the pellet containing the extracted RNA allowed to air-dry before being suspended in 0.01% DEPC-treated H_2O. RNA was quantitated using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) and 2 ug run on a 1.5% formaldehyde gel to confirm its integrity.

6.2.8.2 cDNA conversion

Synthesis of cDNA used 2 µg RNA made up to a volume of 8 µl. One µl random hexamers were added and the samples incubated at 70°C for 10 mins before returning to ice for 5 mins. Samples were combined with 11 µl of the PCR Mastermix to bring the total reaction volume to 20 µl. The PCR conditions were as follows; 10 mins at 25°C, 30 mins at 42°C and 2 mins at 80°C.

6.2.8.3 Quantitative real-time PCR (qRT-PCR)

Quantitative real-time PCR was performed using the StepOne Real-time PCR system (Applied Biosystems, USA). 2-4 µl cDNA was amplified using the KAPA SYBR Fast mastermix (Kapa Biosystems, Cape Town, South Africa) in combination with one of the primer pairs under the conditions stated in the table below (Table 1). Samples were standardised to the house-keeping gene *GusB*. Analysis was carried out using the comparative threshold cycle (C_T) method.

Table 6.1: Primers used for qRT-PCR

Gene	Sequence	Product size	Tm (°C)	No. of cycles
IL-1β Forward	5’ CCACCTCCAGGGACAGGATA 3’	176 bps	58°C	50
IL-1β Reverse	5’ TGGGATCTACACTCTCCAGC 3’			
IL-6 Forward	5’ GGATTCAATGAGGAGACTTGCC 3’	213 bps	58°C	40
IL-6 Reverse	5’ CAGGCTGGCATTTGTGGTTG 3’			
TNF-α Forward	5’ GTAGCCCATGTTGTAGCAAACC 3’	256 bps	58°C	40
TNF-α Reverse	5’ TGATGGCAGAGAGGAGGTTG 3’			
GM-CSF Forward	5’ GACACTGCTGCTGAGATGAATG 3’	173 bps	55°C	40
GM-CSF Reverse	5’ CAGTGCTGCTTGTAGTGGCT 3’			
KPNB1 Forward	5’ CCAGTGCCGAGTGGAATG 3’	191 bps	55°C	40
KPNB1 Reverse	5’ AAATCCCTGACCCTCCTTC 3’			
MMP-2 Forward	5’ TGGCGATGGATACCCCTTT 3’	117 bps	55°C	40
MMP-2 Reverse	5’ TTCTCCCAAGGTCCATAGCTCAT 3’			
MMP-9 Forward	5’ CCGGACCAAGGATACAGTTT 3’	108 bps	60°C	40
MMP-9 Reverse	5’ GCGGTACATAGGGTACATGAG 3’			
TIMP-1 Forward	5’ AGAGACACCAGAGAACCCA 3’	148 bps	55°C	40
TIMP-1 Reverse	5’ TGATGACGAGGTCGGAATTG 3’			
TIMP-2 Forward	5’ CATGATCCCGTGCTACATCTC 3’	105 bps	55°C	40
TIMP-2 Reverse	5’ TTGATGCAGGCGAAGAACT 3’			
COL1A1 Forward	5’ TCTGCGACAACGGCAAGGTG 3’	146 bps	60°C	40
COL1A1 Reverse	5’ GACGCCGGTGGTTTCTTGGT 3’			
COL4A1 Forward	5’ CAGGATGCAATGGCACAACG 3’	115 bps	55°C	40
COL4A1 Reverse	5’ TCACCTGGATCACCCTTCA 3’			
GusB Forward	5’ CTCATTTGGAATTTTGCCGATT 3’	81 bps	55°C/ 58°C	40
GusB Reverse	5’ CCGAGTGAAGATCCCCTTTTTA 3’			

6.2.9 Immunocytochemistry

Cells were cultured on glass coverslips in 6-well plates. Following treatment the cells were fixed in ice-cold 4% paraformaldehyde in PBS for 15 mins at room temperature followed by two washes with cold PBS. Permeabilisation of cells was achieved using 0.25% Triton X-100 in PBS for 10 mins. Cells were washed in PBS, 3x5 mins. Cells were then blocked using 1% BSA in PBST with 0.3 M glycine for quenching, for 30 mins. NFkB p65 primary antibody was used at a dilution of 1:200 in 1% BSA in PBST for 1 hr at RT. Primary antibody was washed off with PBS, 3x5 mins. Secondary antibody, GαR-Cy3, was diluted at a concentration of 1:300 in 1% BSA in PBST and placed on the cells for 1 hr at RT in the dark. Secondary antibody was washed off with PBS, 3x5 mins. DAPI (200 ug/ml) (Sigma-Aldrich, USA) was diluted to 1:400 in PBS and placed on the cells for 5 mins before being washing off with PBS. Coverslips were mounted using Mowial. Fluorescent images were captured at 100x in oil immersion using a Zeiss Axiovert 200M fluorescent microscope with AxioVision 4.8 Zeiss software and an AxioCam HRm (Carl Zeiss, Jena, Germany).

6.2.10 Actin staining

Cells were cultured on glass cover slips in 6-well plates. Following treatment for 24 hours with 5 µM INI-43 the cells were fixed in ice-cold 4% paraformaldehyde in PBS for 15 mins at room temperature. The cells were washed twice with cold PBS and permeabilised in 0.1% Triton X-100 in PBS for 5 mins followed by another two PBS washes. Cells were blocked in 1% BSA in PBS with 0.1M glycine for quenching for 30 mins. Cells were then incubated in 50 ng/ml Phalloidin-Tetramethylrodamine B isothiocyanate (Sigma-Aldrich, USA) in 1% BSA in PBS for 30 mins followed by two PBS washes. The nuclei were stained using DAPI (200 ug/ml) (Sigma-Aldrich, USA) diluted to 1:400 in PBS and placed on the cells for 5 mins before being washed

off with PBS. Coverslips were mounted using Mowial. Fluorescent images were captured at 100x in oil immersion using a Zeiss Axiovert 200M fluorescent microscope with AxioVision 4.8 Zeiss software and an AxioCam HRm (Carl Zeiss, Jena, Germany). ImageJ software was used to measure changes in cell size while the number of processes per cell were counted by eye and averaged for each condition.

6.2.11 Protein harvest and quantification

6.2.11.1 Whole cell lysates from cultured cells

Following relevant treatment of cells in 35 mm or 60 mm tissue culture dishes protein was harvested on ice in RIPA buffer containing a fresh mixture of complete protease inhibitors (Roche, Basel, Switzerland) and 0.1 M Sodium Orthovanadate to inhibit phosphatase activity. A cell scraper was used to remove the cell lysate from the tissue culture dish. Samples were sonicated for 10 seconds before undergoing centrifugation at 10000 x G for 10 mins to remove any cellular debri. The supernatant was stored at -80°C until further use.

6.2.11.2 Nuclear and cytoplasmic fractionation

Separate cytoplasmic and nuclear proteins were extracted to analyse protein expression in individual cellular compartments. Cells were plated at a density of 250 000 cells/ 60 mm plate and treated accordingly before being removed by trypinisation as detailed previously and the cells pelleted by centrifugation. Protein was extracted and fractionated using either the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions or our in-house fractionation protocol as detailed here. The cell pellets were resuspended in Harvest Buffer on ice for 5 minutes,

followed by centrifugation at 1000 x G for 10 minutes which separated the cytoplasmic fraction (supernatant) and the nuclear fraction (pellet). The cytoplasmic fraction was centrifuged again at 14 000 x G for 15 minutes, and the supernatant stored at -80°C. The nuclear pellet was washed in Buffer A, and centrifuged at 1000 x G for 5 minutes. The pellet was then resuspended in Buffer C and vortexed for 15 minutes at 4°C to extract the nuclear protein, followed by centrifugation at 14 000 x G for 10 minutes. The supernatant containing the nuclear protein was collected and stored at -80°C.

6.2.11.3 BCA assay

All protein samples were quantified in 96-well plates using the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacturer's instructions. The absorbencies at OD_{595nm} were determined using a Biotek microplate spectrophotometer (Winooski, VT, USA), alongside the BSA protein standards. A standard curve was plotted using BSA standards and the sample protein concentration calculated. Equal amounts of protein were made up using H_2O and 4x Loading dye to obtain equal volumes.

6.2.12 Western Blot analysis

6.2.12.1 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and protein transfer

Equal amounts of protein (10-30 µg) were loaded into a 10-15% SDS polyacrylamide gel and run in 1x running buffer at 180 V for up to 70 mins. A broad range protein ladder was loaded (5 µl) to determine size of protein bands (Fig. A1.1). Proteins were transferred to a Hybond-

ECL nitrocellulose membrane (AEC-Amersham, Johannesburg, South Africa) in 1x transfer buffer for 1 hour at 100 V.

6.2.12.2 Immunoblotting and chemiluminescent detection

Membranes are then blocked in 5% non-fat dried milk in TBS-T for 1 hour before the primary antibody was applied at its relevant dilution (Table 2) to the membrane overnight at 4°C. After three 10 min washes in TBS-T the secondary antibody was applied for 1 hour at room temperature. Membranes were again washed three times for 10 mins each before protein were detected using Lumiglo (KPL, Inc., Gaithersburg, MD, USA) or Clarity™ Western ECL Substrate (Biorad, Hercules, CA, USA) depending on the strength of the signal. The chemiluminescent signal was detected by exposing the membrane to X-ray film (AGFA, Mortsel, Belgium), followed by immersing the film in developer (AGFA G128), water, then fixative (AGFA G333C) and water.

Table 6.2: Antibodies, concentrations and incubation conditions

Primary Antibody	Conditions	Secondary Antibody	Conditions	Detection
Anti-p-cJUN	1:1000 in 5% BSA	GαR	1:5000 in 5% milk	Biorad Clarity
Anti-cJUN	1:1000 in 5% BSA	GαR	1:5000 in 5% milk	Lumiglo
Anti-NFkB p65	1:5000 in 5% milk	GαR	1:5000 in 5% milk	Biorad Clarity
Anti-NFkB p50	1:5000 in TBS-T	GαR	1:5000 in 5% milk	Biorad Clarity
Anti-KPNB1	1:1000 in TBS-T	GαR	1:5000 in TBS-T	Lumiglo
Anti-TBP	1:500 in TBS-T	GαR	1:5000 in TBS-T	Lumiglo
Anti-B-tubulin	1:1000 in TBS-T	GαR	1:5000 in 5% milk	Lumiglo
Anti-GAPDH	1:5000 in TBS-T	GαM	1:5000 in 5% milk	Lumiglo

6.2.12.3 Stripping and re-probing immunoblots

Membranes were stripped of bound antibodies by submerging the membrane in 10 ml Stripping buffer for 14 mins turning the membrane around halfway. The buffer was neutralised using 1 ml 1 M Tris-EDTA pH 7.5, the membrane was then washed using TBS-T and blocked and probed with a new primary antibody as detailed above.

6.2.13 Electromobility shift assay

6.2.13.1 Biotin-labelling of DNA probe

The wild-type NFkB oligonucleotide used as a probe for direct binding studies was as follows; 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCAACTCCCCTGAAAGGGTCCG-5'. 1 µM forward and reverse probe was labelled using the Biotin 3' End Labelling Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Equal parts of labelled probe were annealed under the following conditions; 3 mins- 95°C, 10 mins- 65°C, 60 mins- 37°C and 1 min- 25°C.

6.2.13.2 Supershift and controls

For the supershift, 2 µl NFκB X p65/p50 antibody was added to the binding reaction. For the controls 1 µl 50 µM unlabelled double-stranded wild-type oligonucleotide or unlabelled double-stranded mutant oligonucleotide, 5'-AGTTGAGGCGACTTTCCCAGGC-3' and 3'-GCCTGGGAAAGTCGCCTCAACT-5', was added to the binding reaction. The incubation at room temperature prior to adding labelled oligonucleotide was extended to 20 mins.

6.2.13.3 Gel electrophoresis and membrane transfer

The binding reaction contained; 5 µg nuclear protein, 5x Incubation buffer, poly DI/DC and 5 µl biotin-labelled double-stranded NFκB oligonucleotide. The reaction was incubated at room temperature for 10 mins without the labelled oligonucleotide and a further 30 mins on ice with the oligonucleotide. DNA loading dye was added to the samples, loaded into a polyacrylamide gel and run at 200 V for 60 mins in 0.5x TBE on ice. DNA-protein complexes were transferred to a N^+ Hybond membrane (AEC-Amersham, Johannesburg, South Africa) at 100 V in 0.5x TBE for 30 mins on ice. The membrane was cross-linked using the Spectrolinker™ XL-1000 (Thomas Scientific, Swedesboro, NJ, USA).

6.2.13.4 Chemiluminescent detection

Protein-DNA complexes were detected on the membrane using the Chemiluminescent Nucleic Acid Detection Kit (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) as per the manufacturer's instructions.

6.2.14 Large-scale Plasmid preparation

To produce adequate quantities of luciferase plasmids for experimental procedures the Qiagen MaxiPrep Kit (Hilden, Germany) was used for large-scale production. Plasmids were first transformed by adding 50 ng plasmid DNA to 30 µl JM109 competent cells (Promega, USA). The mixture was incubated on ice for 20 mins, at 42°C for 2 mins and then on ice again for 2 mins before being transferred to 450 µl Luria Broth and incubated at 37°C for 1 hr while shaking at 150 rpm. The transformation mix (100 µl) was plated onto Ampicillin-selective Luria Broth agar plates and incubated overnight at 37°C. A white colony was selected from the agar

plate and inoculated into 5 ml Luria Broth containing 60 µg/ml Ampicillin and incubated for 8 hrs at 37°C while shaking at 200 rpm. 0.5 ml culture was transferred to 100 ml Luria Broth containing 100 ug/ml Ampicillin and incubated for a further 16 hrs at 37°C while shaking. Bacterial cultures were then harvested by centrifugation at 6000 x G and resuspended in Buffer P1 containing 100 ug/ml RNAse. Resuspended pellets were lysed in Buffer P2 for 5 mins at room temperature after which the solution was neutralised using Buffer P3 for 20 mins on ice. The supernatant was isolated firstly by centrifugation at 20000 x G for 30 mins followed by a second centrifugation step of 15 mins to ensure removal of all suspended material (including genomic DNA, proteins and cell debri). A Qiagen-tip 500 was washed using Buffer QBT, according to the manufacturer's instructions, and the supernatant applied to the tip. Following various washes with Buffer QC the DNA was eluted off the tip using Buffer QF and precipitated using 0.7 volumes isopropanol and centrifuged at 15000 x G for 30 mins. The pellet was washed a final time with 70% ethanol and allowed to air dry before being resuspended in TE buffer, pH 8.0. The DNA concentration was measured using the NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). To confirm isolation of the correct plasmids, plasmids were cut by digestion with restriction enzymes and electrophoresed on an agarose gel. A DNA ladder was loaded alongside the samples in the agarose gel to allow measurement of DNA fragment size in base pairs (Fig. A1.2).

6.2.15 Luciferase assays

Cells were seeded and transfected with the luciferase reporter plasmid in a 24-well plate as detailed earlier. Following relevant treatments to inhibit KPNB1 or transcription factor activity cells were lysed in 100 µl 1x Passive lysis buffer (Promega, USA). Luciferase firefly activity was assayed using the Dual Luciferase kit (Promega, USA) on the Glomax 96 microplate luminometer (Promega, USA). Promoter activity was normalised to Renilla luciferase activity.

6.2.16 Cytometric Bead Array (CBA)

Cells were plated at a density of 150 000 cells/ well in a 6-well plate. Cells were treated with INI-43 and stimulated with PMA before treatment was removed and 1 ml fresh medium was placed on the cells to condition for 16 hrs. Conditioned media was collected, centrifuged at 600 x G for 10 mins at 4°C to remove any cellular debris and stored at -80°C until use. A BD™ Cytometric Bead Array (CBA) (BD Biosciences, USA) was used to measure inflammatory cytokine levels in conditioned media specifically using the Human Inflammatory Cytokine Kit. Samples and standards were prepared according to the manufacturer's instructions and assayed on the BD Accuri C6 Flow Cytometer (BD Biosciences, USA). Cytokine levels were quantified using a standard curve and the results analysed using FlowJo software (Ashland, Oregan, USA).

6.2.17 *In vivo* tumourigenesis assay

Cervical cancer cells, CaSki, were used in the tumourigenesis assay. Cells (5×10^6) suspended in PBS were subcutaneously injected into the hind flank of each nude mouse. Drug treatment commenced once tumours were palpable and the mice randomly divided into two groups of six mice each. The vehicle control group was administered DMSO while the treatment group received INI-43 both at a concentration of 50 mg/kg every 2-3 days for 4 weeks. Throughout the study mice were weighed daily as a measure of health and tumours measured using a calliper twice a week. Tumour size was calculated using the following formula: *tumour volume* $(mm^3) = (length \times width \times width)/2$. At the end of the study mice were euthanised after which the tumours were extracted, weighed, photographed and formalin-fixed for further immunohistochemical analysis.

6.2.18 Immunohistochemistry

6.2.18.1 Sample processing

Formalin-fixed tumours were processed in the Leica TP1020 Processor (Wetzlar, Germany). Specimens were paraffin-wax embedded. A Leica microtome was used to cut 1-2 µm sections which were floated onto APS-coated slides (Marienfield, Germany). Slides were heat-fixed at 60°C for 30 mins.

6.2.18.2 Haemotoxylin and Eosin staining

Slides were dewaxed in xylol and rehydrated through a decreasing ethanol gradient. Samples were placed into Haematoxylin for 9 mins, rinsed in water, washed in Scott's water and placed in Eosin for 3 mins. Slides were dehydrated in an increasing ethanol gradient, placed in xylol and mounted in resin (Entellan, Merck, Darmstadt, Germany).

6.2.18.3 Immunoperoxidase staining

Slides were dewaxed in xylol and rehydrated through a decreasing ethanol gradient. Samples were blocked for endogenous peroxidase activity using 3% Hydrogen peroxide in water for 15 mins. Antigen retrieval was achieved using 0.01 M citrate buffer pH 6.0 for 2 mins (KPNB1) or 1 mM EDTA, pH 8.0 for 1 min 30 secs (KI-67) in a pressure cooker. Slides were washed with PBS-T in between steps. KPNB1 primary antibody, 1:100 in PBS, was placed onto the samples for 2 hrs at room temperature while KI-67 primary antibody, 1:100 in PBS, was placed on the samples for 1 hr at room temperature. Envision polyclonal rabbit secondary antibody (KPNB1) or Envision monoclonal mouse secondary antibody (KI-67) was placed onto the samples for

30 mins. The chromogen DAB (Dako, Glostrup, Denmark) was used for 8 mins to develop the peroxidase colour while 10 mins in 1% copper sulphate enhanced the colour. Haematoxylin was used as a counter stain and slides were dehydrated across an increasing ethanol gradient before being placed in xylol and mounted using in resin (Entellan, Merck, Darmstadt, Germany). All samples were assessed and scored by a professional pathologist, Prof Dhiren Govender, UCT Division of Anatomical Pathology.

6.2.19 Statistical analysis

Experiments were performed in triplicate or quadruplicate and expressed as the mean ± standard error of the mean (SEM), unless otherwise stated. Experiments were repeated at least two independent times. For data analysis a two-tailed Student's t-test was used where a p-value of <0.05 was considered statistically significant. Statistical analysis was performed using GraphPad Prism V5.0 (GraphPad software, San Diego, California, USA).

6.3 SOLUTIONS

6.3.1 Tissue culture solutions

Complete Media

500 ml DMEM

50 ml FCS (10%)

5 ml PenStrep (1%)

10x PBS

40 g NaCl

1 g KCl

3.82 g $Na_2HPO_4.2H_2O$

2.1 g KH_2PO_4

Up to 500 ml dH_2O

Cryopreservation Media

20% FCS

10% DMSO

70% Complete media

Solubilisation Solution

25 g SDS

76.6 ul conc. HCL

Up to 250 ml with dH_2O

MTT

0.1 g MTT

20 ml 1x PBS

6.3.2 Gelatin Zymography solutions

4x Sample buffer

2.5 ml 1 M Tris pH 6.8

3 ml 20% SDS

0.5 ml 0.1% Bromophenol Blue

4 ml Glycerol

Separating Gel

2.26 ml dH_2O

1.25 ml 1% gelatin

4.16 ml 30% Acrylamide/Bis-acrylamide

4.7 ml 1 M Tris-HCL buffer, pH 8.8

62.5 ul 20% SDS

37.5 ul 10% AMPS

7.5 ul TEMED

Stacking Gel

2.625 ml dH_2O

500 ul 30% Acrylamide/Bis-acrylamide

625 ul 1 M Tris-HCL buffer, pH 6.8

25 ul 20% SDS

18.76 ul 10% AMPS

2.5 ul TEMED

10x Running Buffer

30.29 g Tris

144 g Glycine

pH 8.3

Up to 1 L with dH_2O

1x Running Buffer

5 ml 20% SDS

100 ml 10x Running Buffer

895 ml dH_2O

10x Renaturing Solution

25% Triton-X100 in dH_2O

10x Developing Buffer

500 ml 1 M Tris-HCL, pH 7.8

83.98 g NaCl

5.5 g $CaCl_2$

2 g Brij-35

Staining Solution

5 g Coomassie Blue R-250

50 ml Methanol

100 ml Acetic Acid

Destaining Solution

50 ml Acetic Acid

100 ml Methanol

850 ml dH_2O

6.3.3 RNA solutions

DEPC dH_2O

100 ul DEPC

1 L dH_2O

RNA integrity gel

0.75 g Agarose

5 ml 10x MOPS

42 ml dH_2O

2.7 ml Formaldehyde (37%)

2.5 ul Ethidium bromide

10x MOPS

41.86 g MOPS

16.6 ml 3 M Sodium Acetate

20 ml 0.5 M EDTA

pH 7.0

RNA loading dye

720 ul Formamide

160 ul 10x MOPS

260 ul Formaldehyde (37%)

40 ul dH_2O

100 ul 80% Glycerol

200 ul 0.25% Bromophenol blue

PCR Mastermix

4 ul 5x Reaction buffer

1.6 ul $MgCl_2$

2 ul 10 nM dNTPs

1 ul RNAsin

1 ul Reverse transcriptase

1.4 ul DEPC dH_2O

6.3.4 Immunocytochemistry solutions

16% Paraformaldehyde

16 g Paraformaldehyde

Up to 80 ml dH_2O

PBS-T

1 L 1x PBS

500 ul Tween-20

Fixative

2.5 ml 16% Paraformaldehyde

1 ml 10x PBS

6.5 ml dH_2O

Permeabilisation solution

0.125 ml Triton X-100

49.875 ml PBS

Blocking Solution

0.5 g BSA

1.126 g Glycine (0.3 M)

Up to 50 ml PBS-T

Mowial

9.6 g Moviol 4-88

24 ml Glycerol

24 ml dH_2O

48 ml 0.2 M Tris, pH 8.5

Anti-fade: Add few grains n-propyl gallate to 1 ml Mowiol (prepare fresh)

6.3.5 Protein solutions

RIPA Buffer

3 ml 5 M NaCl

1 ml Triton X-100

1 g Sodium deoxycholate

1 ml 10% SDS

1 ml 1 M Tris pH 7.4

94 ml dH_2O

Harvest Buffer

10 mM HEPES pH 7.9

50 mM NaCl

0.5 M Sucrose

0.1 mM EDTA

0.5% Triton X-100

Add fresh PI and Na_2VO_5

Buffer A

10 mM HEPES pH 7.9

10 mM KCl

0.1 mM EDTA

0.1 mM EGTA

Add fresh PI and Na_2VO_5

Buffer C

10 mM HEPES pH 7.9

500 mM NaCl

0.1 mM EDTA

0.1 mM EGTA

0.1% NP40

Add fresh PI and Na_2VO_5

10x TBS

60.5 g Tris

87.5 g NaCl

Up to 1 L dH_2O

pH 7.5

TBS-T

100 ml 10xTBS

900 ml dH_2O

500 ul Tween-20

10x Transfer Buffer

72 g Glycine

19 g Tris

Up to 500 ml with dH_2O

1x Transfer Buffer

100 ml 10x Transfer Buffer

200 ml Isopropanol

700 ml dH_2O

10x Running Buffer

20 g Glycine

31.6 g Tris

50 ml 10% SDS

Up to 500 ml dH_2O

4x Loading dye

2.5 ml 1 M Tris pH 6.8

3 ml 20% SDS

0.5 ml 0.1% Bromophenol Blue

4 ml Glycerol

50 ul β-mercaptoethanol/0.5 ml

10% Resolving Gel

2.85 ml dH_2O

3.75 ml 1 M Tris pH 8.8

3.3 ml 30% Acrylamide/Bis-acrylamide

50 ul 10% SDS

175 ul 10% AMPS

17.5 ul TEMED

15% Resolving Gel

1.55 ml dH_2O

3.75 ml 1 M Tris pH 8.8

5 ml 30% Acrylamide/Bis-acrylamide

50 ul 10% SDS

175 ul 10% AMPS

17.5 ul TEMED

4% Stacking Gel

3.65 ml dH_2O

0.625 ml 1 M Tris pH 6.8

0.65 ml 30% Acrylamide/Bis-acrylamide

50 ul 10% SDS

60 ul 10% AMPS

6 ul TEMED

Stripping Buffer

37.54 g Glycine

Up to 500 ml with dH_2O

pH 2.5

6.3.6 Electromobility shift assay solutions

5x Incubation Buffer

100 mM HEPES, pH 7.9

250 mM KCl

2.5 mM DTT (Add fresh)

1 mM EDTA

5 mM $MgCl_2$

20% Ficoll 400

Gel

5.4 ml 30% Acrylamide/Bis-acrylamide

2 ml 10x TBE

13.8 ml dH_2O

200 ul 10% AMPS

10 ul TEMED

6.3.7 Bacterial solutions

Luria Broth

5 g Tryptone

2.5 g Yeast Extract

2.5 g NaCl

500 ml dH_2O

Luria Agar

2.5 g Tryptone

1.25 g Yeast Extract

2.5 g NaCl

3.75 g Agar

Resuspension Buffer (P1)

6.06 g Tris

3.72 g $Na_2EDTA.2H_2O$

800 ml dH_2O

pH 8.0

100 mg/l RNAse A

Lysis Buffer (P2)

8.0 g NaOH

50 ml 20%SDS

950 ml dH_2O

Neutralisation Buffer (P3)

294.5 g Potassium acetate

500 ml dH_2O

pH 5.5 (Glacial Acetic Acid)

Equilibration Buffer (QBT)

43.83 g NaCl

10.46 g MOPS

800 ml dH_2O

pH 7.0

150 ml Isopropanol

15 ml 10% Triton X-100

Wash Buffer (QC)

58.44 g NaCl

10.46 g MOPS

800 ml dH_2O

pH 7.0

150 ml Isopropanol

Elution Buffer (QF)

73.05 g NaCl

6.06 g Tris

800 ml dH_2O

pH 8.5

150 ml Isopropanol

6.3.8 DNA solutions

TE Buffer

1 ml 1 M Tris pH 8.0

0.2 ml 0.5 M EDTA pH 8.0

98.8 ml dH_2O

pH 8.0

10x TBE

108 g Tris

55 g Boric Acid

7.4 g EDTA

Up to 1 L dH_2O

1% DNA gel

0.5 g Agarose

50 ml 1x TBE

2.5 ul Ethidium Bromide

6.3.9 Immunohistochemistry solutions

Mayer's Haemotoxylin

50 g Aluminum potassium sulphate

1 L dH_2O

1 g Haematoxylin

0.2 g Sodium iodate

20 ml Glacial acetic acid

Eosin Y Stock solution

2 g Eosin Y

40 ml dH_2O

160 ml 95% ethanol

Eosin Y Working solution

200 ml Eosin Y stock solution

600 ml 80% ethanol

4 ml glacial acetic acid

10x Citrate Buffer

0.1 M Citrate

Up to 1 L dH_2O

pH 6.0

10x EDTA Buffer

10 mM EDTA

Up to 1 L dH_2O

pH 8.0

APPENDIX I: PROTEIN & DNA LADDERS

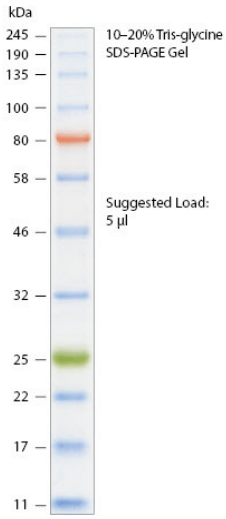


Figure A1.1: Color Prestained Protein Standard, Broad Range (11-245 kDa). Protein ladder used to determine size of protein bands on SDS-PAGE gels of 10-15%.

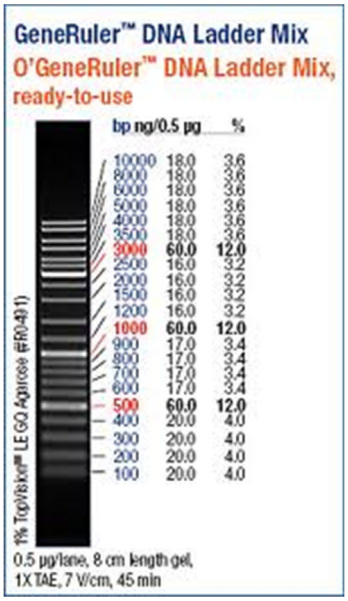


Figure A1.2: O'GeneRuler DNA Ladder Mix (100-10000 bps). DNA ladder used to measure size of DNA fragments on an agarose gel electrophoresis.

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PUBLICATIONS

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Research Paper

KPNB1-mediated nuclear import is required for motility and inflammatory transcription factor activity in cervical cancer cells

Tamara Stelma[1] **and Virna D. Leaner**[1]

[1]Division of Medical Biochemistry and Structural Biology, SAMRC Gynaecology Cancer Research Centre, Department of Integrative Biomedical Sciences, Faculty of Health Sciences, Institute of Infectious Diseases and Molecular Medicine, University of Cape Town, Cape Town, South Africa

Correspondence to: *Virna D. Leaner,* ***email:*** *Virna.Leaner@uct.ac.za*

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ABSTRACT

Karyopherin β1 is a nuclear import protein involved in the transport of proteins containing a nuclear localisation sequence. Elevated Karyopherin β1 expression has been reported in cancer and transformed cells and is essential for cancer cell proliferation and survival. Transcription factors such as NFκB and AP-1 contain a nuclear localisation sequence and initiate the expression of multiple factors associated with inflammation and cancer cell biology. Our study investigated the effect of inhibiting nuclear import via Karyopherin β1 on cancer cell motility and inflammatory signaling using siRNA and the novel small molecule, Inhibitor of Nuclear Import-43, INI-43. Inhibition of Karyopherin β1 led to reduced migration and invasion of cervical cancer cells. Karyopherin β1 is essential for the translocation of NFκB into the nucleus as nuclear import inhibition caused its cytoplasmic retention and decreased transcriptional activity. A similar decrease was seen in AP-1 transcriptional activity upon Karyopherin β1 inhibition. Consequently reduced interleukin-6, interleukin-1 beta, tumour necrosis factor alpha and granulocyte macrophage colony stimulating factor expression, target genes of NFkB and AP-1, was observed. Migration studies inhibiting individual transcription factors suggested that INI-43 may affect a combination of signaling events. Our study provides further evidence that inhibiting KPNB1 has anti-cancer effects and shows promise as a chemotherapeutic target.

INTRODUCTION

The Karyopherin beta superfamily forms the major class of soluble transport proteins that transport proteins larger than 20-40kDa through the nuclear pore complex (NPC). Karyopherin β1 (KPNB1) is the predominant nuclear import protein and transports cargo into the nucleus independently (non-classical pathway) or through the association with a karyopherin adaptor protein (classical pathway) [1]. In the classical import pathway cargo is identified by a Karyopherin alpha protein through a nuclear localisation sequence (NLS) and binds forming a dimer. Together this dimer then binds KPNB1 and is allowed access through the NPC as a trimeric complex [2–4]. In the non-classical pathway KPNB1 is able to directly recognise a specific NLS on the cargo, binding to it. The nuclear transport system plays a critical role in normal cell functioning and therefore it isn't unusual that its dysregulation has been associated with carcinogenesis. KPNB1 expression has been found to be upregulated in various cancers including cervical cancer and correlates with a poor patient prognosis in gastric cancer [5, 6].

One of the classical hallmarks of cancer is the ability of cancer cells to invade tissue surrounding the site of the primary tumour and metastasize to a secondary location [7]. This occurrence is responsible for the majority of cancer-related deaths. When Hanahan and Weinberg (2011) revisited the classical hallmarks they noted that an inflammatory microenvironment plays an important role in sustaining many of the other hallmarks of cancer including the ability of cancer cells to invade [8]. The presence of chemokines and cytokines in the

tumour microenvironment, whether produced by cancer cells or infiltrating immune cells, directly contribute to the activation of transcription factors such as NFkB and AP-1 [9–11]. TNF-α is a cytokine commonly known to activate both NFkB and AP-1 [12]. The activation of such transcription factors initiates the expression of various target genes contributing to cancer progression by enhancing proliferation (IL-1, TNF-α, GM-CSF, Cyclin D1), evading apoptosis (BCL2, TRAF2, BCL3) and promoting cell invasion (IL-1, IL-6, TNF, MMPs) [13–20].

The NFκB family consists of 5 members of which p65 and p50 are activated in the canonical activation pathway in an inflammatory response. Activation and translocation of the transcription factor into the nucleus can be induced in most cell types but some cells such as macrophages and tumour cells can have constitutively active NFκB. Activation can be stimulated by several events including the presence of cytokines or artificially in response to the phorbol ester, phorbol-12-myristate-13-acetate (PMA) [21, 22]. It has been identified that NFκB's p65 and p50 subunits have a classical NLS, suggesting that they require KPNB1 for their nuclear import [23]. No mutations in the NFκB gene or its inhibitors have been associated with cancer but rather the increased presence of activated NFκB has been associated with an increase in inflammation promoting carcinogenesis [10, 24–27].

Activator protein 1 (AP-1) is a transcription factor consisting of dimers of mainly JUN and FOS family members. Much like NFkB it is activated by inflammatory cytokines and growth factors as well as the phorbol ester, PMA. AP-1 regulates genes involved in many cellular processes including; proliferation, differentiation, apoptosis, angiogenesis and tumour invasion. Increased expression of JUN and FOS family members is associated with several cancers [28–30]. Nuclear import of AP-1 is suggested to be mediated through the non-classical nuclear import pathway as KPNB1 was found to have a significantly greater affinity for AP-1 than Karyopherin alpha and therefore is reported to be transported into the nucleus by KPNB1 alone [31].

As NFkB and AP-1 play such a pivotal role in inflammatory signaling and promoting the cancer phenotype, inhibiting their function could have chemotherapeutic benefits [32–36]. The small molecule inhibitor of KPNB1, INI-43, has been described by van der Watt *et al.* (2016) to interfere with nuclear import of transcription factors such as NFAT. Nuclear import inhibition through KPNB1 has also previously been shown in our laboratory and by others to have anti-cancer potential by inhibiting cancer cell proliferation and inducing cell death by apoptosis *in vitro* as well as inhibiting tumour growth *in vivo* [5, 37–39]. As both NFκB and AP-1 require KPNB1 for their nuclear translocation we hypothesized that inhibiting KPNB1 could inhibit the activity of these transcription factors. Thereby blocking the expression of inflammatory target genes which contribute to the invasive potential of cancer cells. In this study we investigated the effects of inhibiting KPNB1 using siRNA and a novel small molecule, INI-43, on cancer cell migration and invasion as well as NFkB and AP-1 transcriptional function.

RESULTS

KPNB1 is required for the migration and invasion of cervical cancer cells

In our previous studies we have reported that KPNB1 is required for the survival and proliferation of cervical cancer cells and that inhibition of its expression and activity resulted in cell death via apoptosis [5, 37]. Little is known about the role of KPNB1 in other cancer phenotypes such as migration and invasion. In this study, we investigated the requirement of KPNB1 expression and activity for cancer cell migration and invasion. Using transwell migration assays, we showed that knocking down KPNB1 using siRNA reduced the ability of HeLa cervical cancer cells to migrate through the transwell membrane in the presence of PMA stimulation (Figure 1A). Quantification of the migration of control and KPNB1 siRNA treated cells shows a significant reduction in migration when KPNB1 expression is inhibited (Figure 1B). The small molecule inhibitor of nuclear import, INI-43, was also able to reduce the migratory ability of HeLa and SiHa cervical cancer cells (Figure 1C). Quantification of migratory ability following INI-43 treatment shows significant reductions in migration in both cervical cancer cell lines (Figure 1D). The invasive ability of cervical cancer cells was assessed using a transwell invasion plate with matrigel-coated chambers. The inhibition of KPNB1 by both siRNA (Figure 2A & 2B) and INI-43 (Figure 2C & 2D) significantly interfered with both HeLa and SiHa cancer cell invasion. As these assays assessed the role of nuclear import inhibition of cancer cell motility, the effect of cell death was eliminated by optimizing treatment duration and normalizing the results to concurrent live cells as measured using the MTT cell proliferation assay. The ability of cancer cells to invade the extracellular matrix is dependent on the activity of matrix metalloproteases (MMPs). We therefore assessed gelatinase activity of MMP-9 using a gelatin Zymography and looked at MMP-2, TIMP-1 and TIMP-2 expression using qRT-PCR following nuclear import inhibition. Conditioned media was collected from HeLa and SiHa cells following INI-43 treatment and PMA stimulation and the results showed a substantial reduction in MMP-9 activity following nuclear import inhibition (Figure 3A). The expression of MMP-2 was found to be downregulated following INI-43 treatment in HeLa cells (Figure 3B) while the inhibitors of matrix metalloproteases, TIMP-1 and TIMP-2, were significantly upregulated (Figure 3C & 3D). Together these results provide further evidence for the role of KPNB1 in cancer cell biological processes such as migration and invasion.

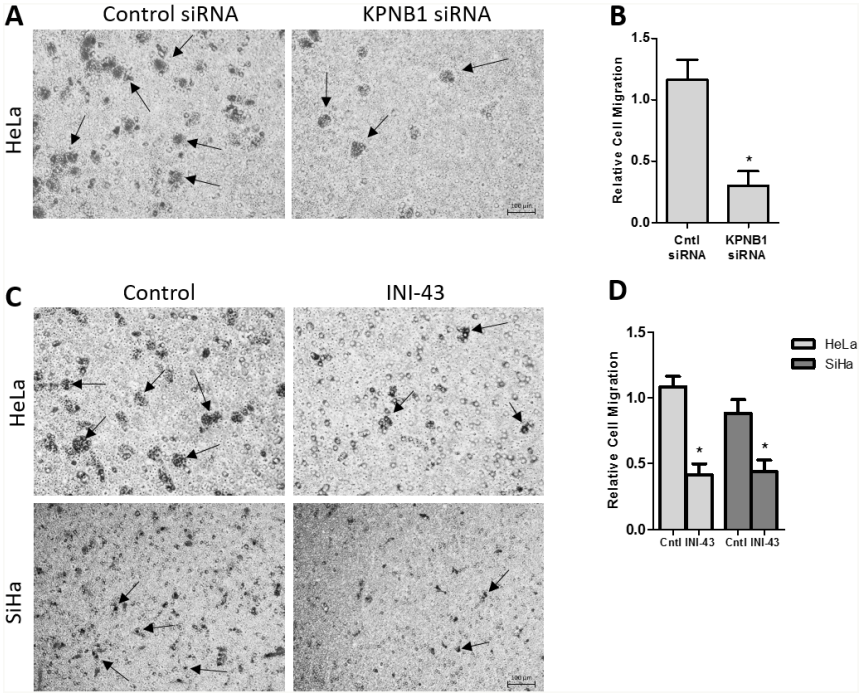

Figure 1: Effect of KPNB1 inhibition on cervical cancer cell migration. **(A)** Representative images from the transwell migration chamber showing migration of HeLa cells following KPNB1 knockdown, arrows identifying migrating cells. Scale bar represents 100 μm. **(B)** Quantification of transwell migration assay following KPNB1 knockdown normalized to MTT cell viability. **(C)** Representative images showing HeLa and SiHa cell migration through the membrane following a 3 hour 10 μM INI-43 pre-treatment, arrows identifying migrating cells **(D)** The number of HeLa and SiHa cells that migrated through the transwell chamber following INI-43 treatment were quantified and normalized to MTT cell viability. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05).

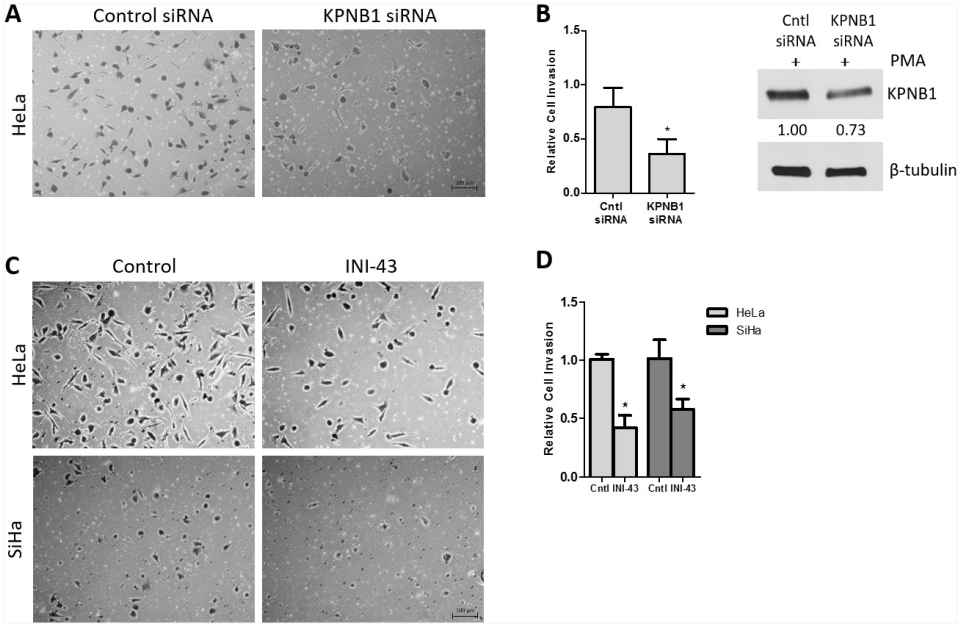

Figure 2: Effect of KPNB1 inhibition on cervical cancer cell invasion. **(A)** Representative images of the invasion transwell assay showing invasion of HeLa cells through the matrigel-coated transwell membrane following KPNB1 knockdown. Scale bar represents 100 μm. **(B)** Quantification of the invasion assay following KPNB1 knockdown normalized to MTT cell viability. Western blot confirmed KPNB1 knockdown. **(C)** Representative images of the invasion assay following 3 hr 10 μM INI-43 pre-treatment of HeLa and SiHa cells showing the number of cells able to invade the matrigel-coated membrane following nuclear import inhibition. **(D)** Quantification of invasion assay following INI-43 treatment, normalized to MTT cell viability. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05).

Inhibition of KPNB1 interferes with NFkB subcellular localisation

Cancer cell proliferation, migration and invasion has a strong association with the correct function of transcription factors that associate with cancer such as NFkB. For NFkB to be active as a transcription factor it is essential that it is transported into the nucleus where it can bind the DNA and initiate transcription of its target genes. Here, we investigated the requirement of the nuclear import protein, KPNB1, for subcellular localisation of the NFkB p65/p50 dimer in cervical cancer cells using multiple techniques including; immunofluorescent analysis, nuclear/cytoplasmic protein separation and electromobility shift assays (EMSA). Translocation of NFkB into the nucleus was stimulated using PMA. Immunofluorescent analysis shows that PMA treatment results in the translocation of NFkB into the nucleus but inhibiting KPNB1 through siRNA transfection or INI-43 pre-treatment blocks nuclear translocation of

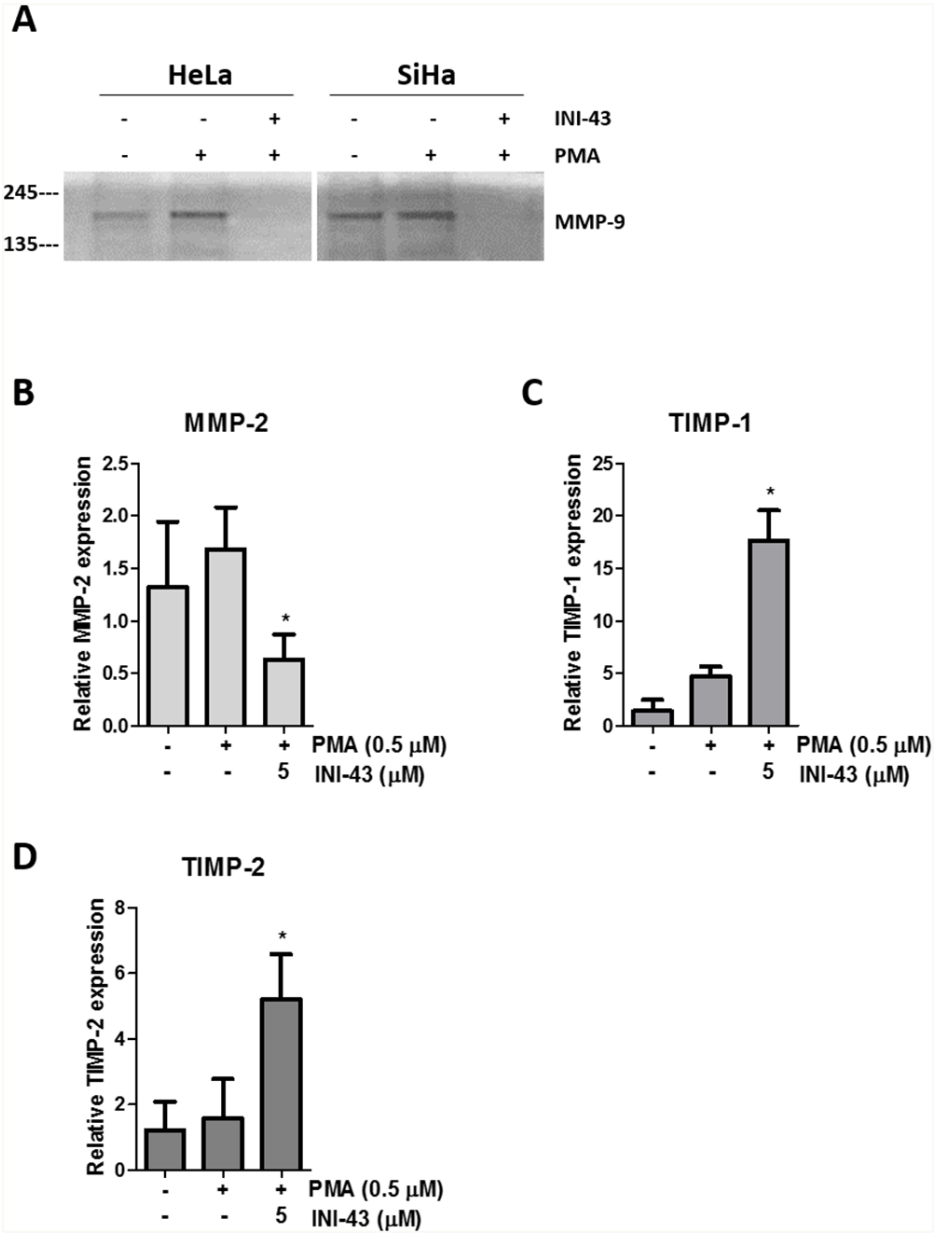

Figure 3: KPNB1 inhibition affects MMP-9 activity and MMP-2, TIMP-1 and TIMP-2 expression. **(A)** Gelatin Zymography showing MMP-9 activity in HeLa and SiHa conditioned media collected after 16 hours following a 3 hour pre-treatment of 10 μM INI-43 and a 1 hour 0.5 μM PMA stimulation. qRT-PCR analysis of mRNA expression following 5 μM INI-43 treatment for 21 hours with the addition of 0.5 μM PMA for a further 3 hours in HeLa cells shown for MMP-2 **(B)** and inhibitors of matrix metalloproteases, TIMP-1 **(C)** and TIMP-2 **(D)**. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05).

NFkB and causes its cytoplasmic retention (Figure 4A). Quantification of fluorescent images shows an overall reduction in nuclear NFkB in KPNB1 inhibited cells following PMA stimulation (Figure 4B). Western blotting was used to validate KPNB1 knockdown (Figure 4C). These findings were independently validated using nuclear/cytoplasmic fractionation of protein samples followed by western blot analysis for the p65 and p50 subunits of NFkB. In the presence of KPNB1 siRNA or treatment with INI-43, a decrease in nuclear and concomitant increase in cytoplasmic NFkB p65 and p50 was observed (Figure 4D). To further elucidate the effect of inhibiting KPNB1 on NFkB, EMSAs were performed to investigate the ability of nuclear NFkB to bind a NFkB consensus sequence. A substantial decrease in the presence of the nuclear NFkB/DNA complex was observed following nuclear import inhibition using KPNB1 siRNA or INI-43 treatment (Figure 5A & 5B). Western blotting was used to confirm protein nuclear/cytoplasmic fractionation and KPNB1 knockdown (Figure 5A). Supershift analysis with antibodies specific to the NFkB p65 and p50 subunits identified NFkB p65 as a protein present in the complex. A reduction in the binding when the supershift was performed using the NFkB p50 antibody suggests competitive binding of the protein with the biotin-labelled oligonucleotide (Figure 5C). Together,

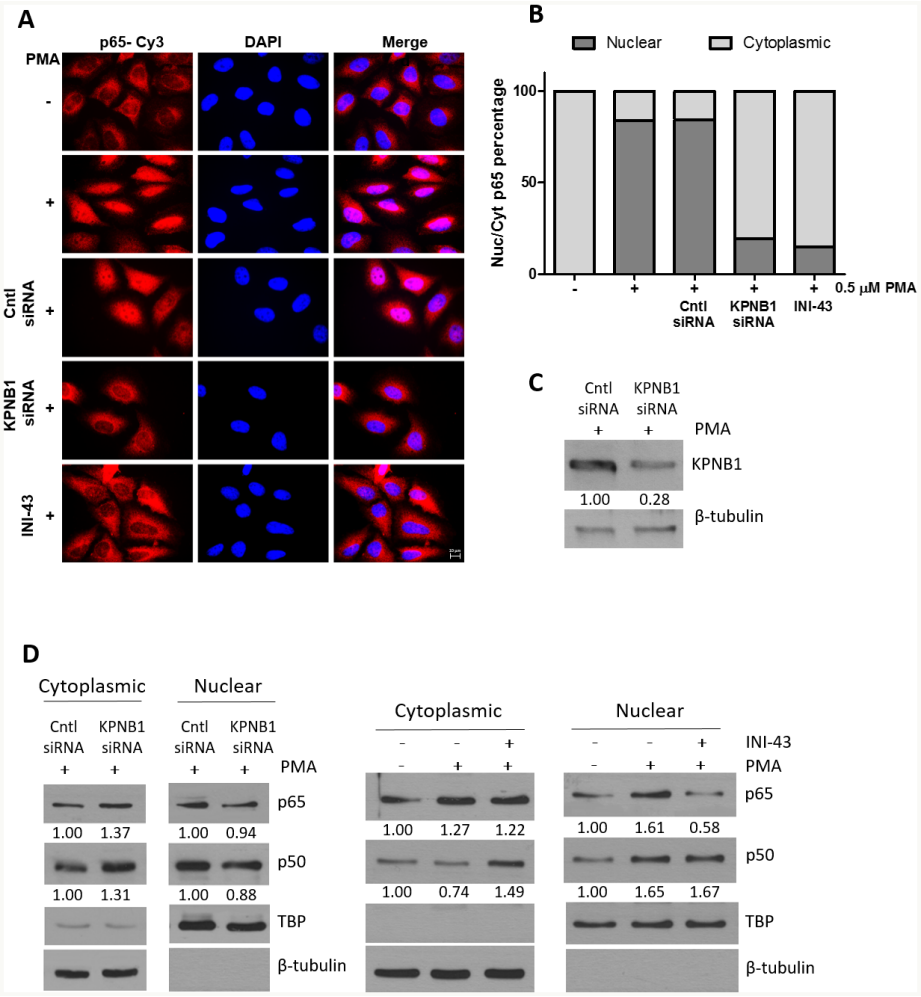

Figure 4: NFkB localisation following KPNB1 inhibition. (A) Representative immunofluorescent images showing NFκB p65 expression and localisation as well as nuclear staining (DAPI) in HeLa cells. The results show the changes in cellular localisation of NFkB following a 1 hour treatment with 0.5 µM PMA, as well as inhibiting KPNB1 through siRNA knockdown or 3 hour pre-treatment with 10 µM INI-43 in addition to PMA stimulation (x100 Objective). Scale bar represents 10 µm. **(B)** Screening of 250 cells over 25 fluorescent images per treatment were scored into "predominantly nuclear" or "predominantly cytoplasmic" p65 fluorescence according to nuclear/cytoplasmic fluorescent intensity analysis. The graph was plotted using the percentage nuclear and cytoplasmic p65 fluorescence. **(C)** Knockdown of KPNB1 was confirmed by western blot. **(D)** Western blot analysis of NFκB p65 and p50 expression in the cytoplasmic and nuclear protein fraction of HeLa cells transfected with KPNB1 siRNA or pre-treated with 10 µM INI-43 for 3 hours before a 1 hour 0.5 µM stimulation with PMA. β-tubulin was used as a cytoplasmic loading control while TBP (TATA-binding protein) was used as a nuclear loading control. Densitometric analysis was normalized to loading controls.

these results confirm that KPNB1 is necessary for NFkB nuclear localisation.

NFkB transcriptional activity and inflammatory target gene expression are inhibited in KPNB1-inhibited cells

Since it was established that NFkB requires KPNB1 for its transport into the nucleus, we postulated that inhibiting KPNB1 should result in a change in NFkB transcriptional activity and NFkB target gene expression. The transcriptional activity of NFkB was quantified using a luciferase reporter assay of both an artificial NFkB consensus binding site containing promoter as well as a known NFkB target gene, the IL-6 promoter-luciferase construct. Our data shows that inhibiting KPNB1 using siRNA resulted in significantly reduced transcriptional activity of the NFkB promoter-luciferase construct (Figure 6A). Treatment of HeLa cells with INI-43 also resulted in a significant reduction of PMA-stimulated NFkB transcriptional activity in a dose-dependent manner to a level which was comparable to the effect of the NFkB inhibitor, JSH-23 (Figure 6B). The IL-6 promoter was similarly inhibited by both KPNB1 siRNA (Figure 6C) and INI-43 treatment (Figure 6D). As inhibiting KPNB1 resulted in an inhibition of NFkB activity we next investigated whether inhibiting nuclear import affects the expression of NFkB target genes specifically involved in inflammatory signaling associated with cell biology changes in cancer. Inflammatory cytokines; IL-6, IL-1β and TNF-α are target genes of NFkB and the expression was monitored in response to KPNB1 inhibition. HeLa cells transfected with KPNB1 siRNA showed not only reduced mRNA expression of the KPNB1 gene but a significant reduction in the mRNA expression of all three inflammatory cytokines (Figure 7A). The small molecule inhibitor, INI-43, had a similar inhibitory effect and significantly inhibited PMA-stimulated IL-6 (Figure 7B),

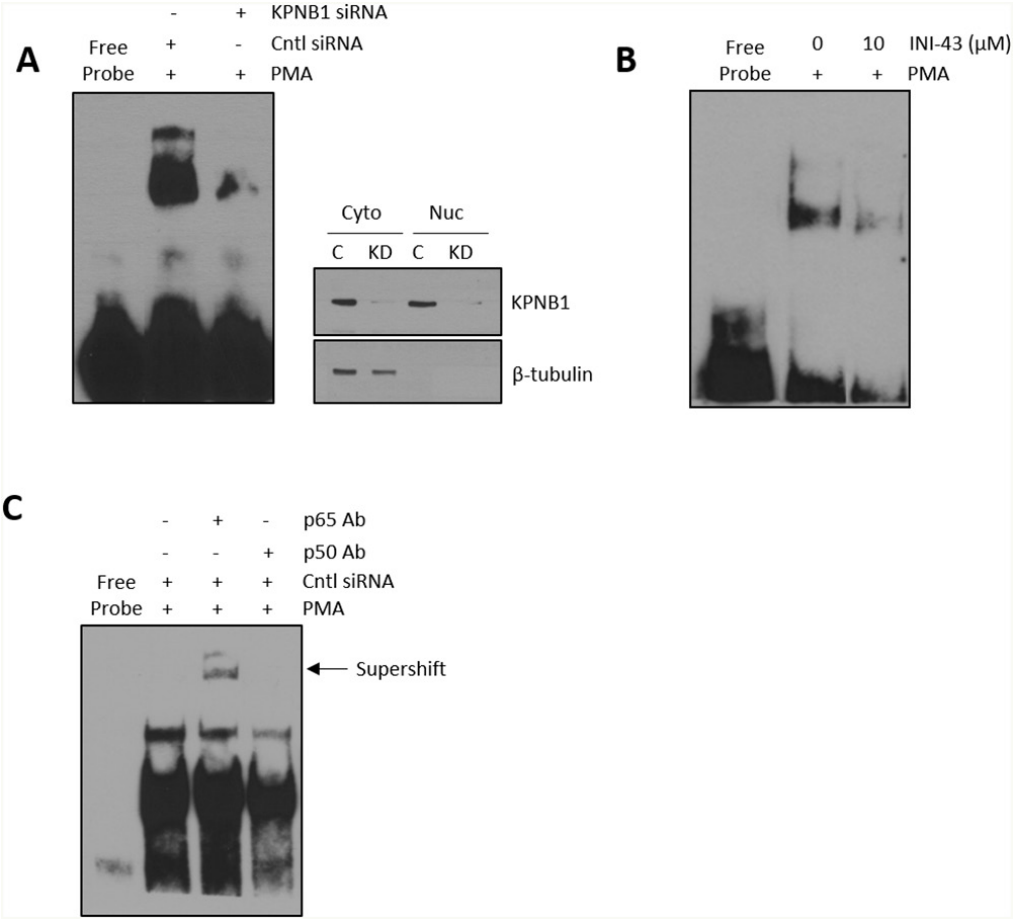

Figure 5: Formation of a nuclear NFkB/DNA binding complex following KPNB1 inhibition. (A) Electromobility shift assay (EMSA) showing binding of nuclear protein to a biotin-labelled NFκB oligonucleotide following KPNB1 siRNA transfection in PMA-stimulated HeLa cells. Western blot analysis of fractionated protein samples confirmed KPNB1 knockdown and fractionation. **(B)** EMSA of nuclear extracts obtained from cells pre-treated with 10 μM INI-43 for 2 hours followed by a 1 hour 0.5 μM PMA stimulation. **(C)** Supershift analysis with the NFκB p65 antibody showing a shift in the DNA-protein complex while the p50 antibody reduced protein binding both confirming the presence of NFkB in the DNA-protein complex.

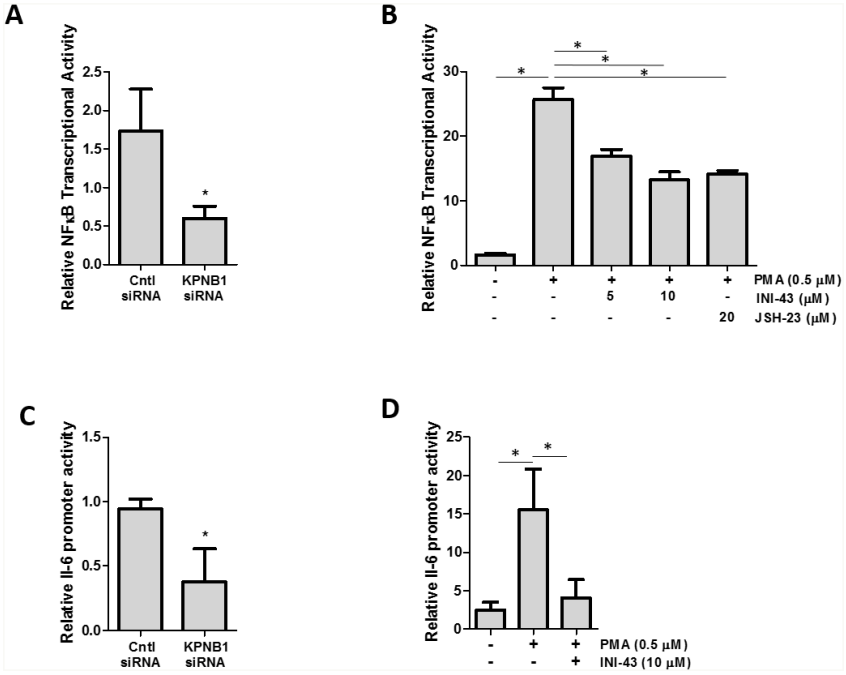

Figure 6: KPNB1 inhibition affects NFkB transcriptional activity. (A) NFκB transcriptional activity using an NFkB binding site-luciferase reporter transiently transfected into HeLa cells following KPNB1 knockdown and stimulation with PMA. **(B)** NFκB transcriptional activity in HeLa cells following a 3 hour 0.5 μM PMA stimulation but subsequently inhibited following a 21 hour pre-treatment with INI-43 and JSH-23 treatment (24 hr total treatment time). **(C)** IL-6 promoter activity shown following KPNB1 knockdown and stimulation with PMA. **(D)** The effect of 0.5 μM PMA stimulation for 3 hours on IL-6 promoter activity is shown in HeLa cells followed by pre-treatment with 10 μM INI-43 for 21 hours. Results shown are the mean ± SD of experiments performed in quadruplicate. (*p<0.05)

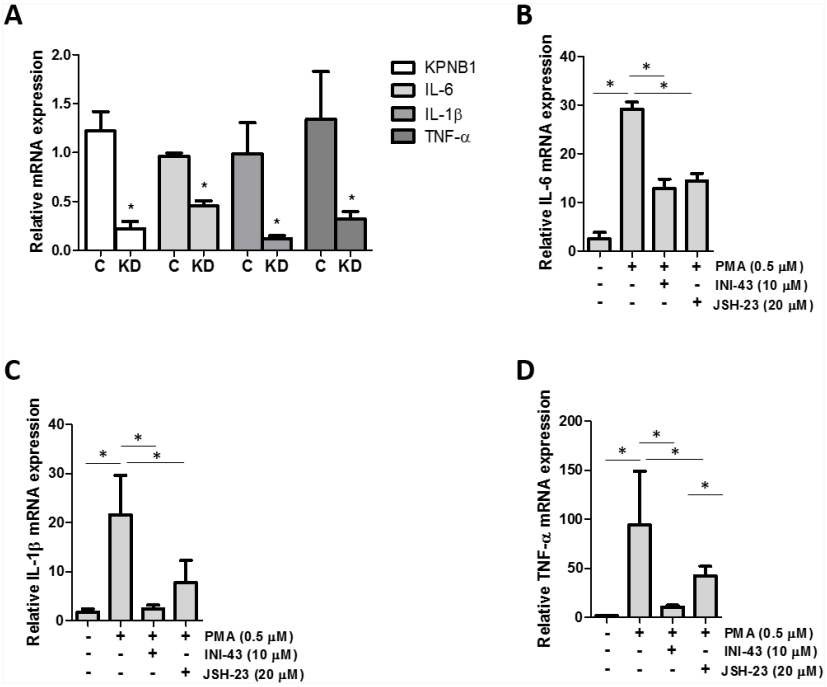

Figure 7: KPNB1 inhibition affects inflammatory NFkB target gene expression. (A) qRT-PCR analysis of mRNA expression showed the effect of KPNB1 knockdown (C= Control, KD= Knockdown) on KPNB1 mRNA expression, confirming knockdown, as well as expression of inflammatory target genes; IL-6, IL-1β and TNF-α. **(B)** IL-6 mRNA expression shown following stimulation for 1 hour with 0.5 μM PMA as well as pre-treatment for 2 hours with 10 μM INI-43 or 23 hours 20 μM JSH-23 followed by 1 hour PMA stimulation. The same conditions were used to look at IL-1β mRNA expression **(C)** and TNF-α **(D)**. Target gene expression was normalized to expression of the housekeeping gene, GAPDH. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05)

IL-1β (Figure 7C) and TNF-α (Figure 7D) gene expression which was comparable to the effect of the NFkB inhibitor, JSH-23. The enhanced reduction of TNF-α mRNA expression in INI-43 treated cells in comparison to the effect of JSH-23 suggests that KPNB1 inhibition by INI-43 on NFkB alone may not be fully responsible for the effect of INI-43 on inflammatory target gene expression (Figure 7D). Other transcription factors such as AP-1 are also known to regulate cytokine expression, namely IL-6, and may too depend on KPNB1 for its activity. Together these results provide evidence that the inhibition of nuclear import via KPNB1 associates with decreased expression of pro-inflammatory cytokine expression.

KPNB1 inhibition affects activity and target gene expression of the AP-1 transcription factor

Transcription factors, other than NFkB, also contain a nuclear localisation sequence (NLS) and may depend on KPNB1 for nuclear entry. The transcription factor AP-1 has been reported to have a NLS present on the c-JUN component, although nuclear import of c-JUN is not limited to KPNB1 only [40]. We investigated whether KPNB1 may also influence activity and target gene expression of the AP-1 transcription factor. The transcriptional activity of AP-1 was quantified using a luciferase reporter construct containing multiple artificial AP-1 binding sites. Our data shows that the small molecule inhibitor of nuclear import, INI-43, was able to significantly reduce PMA-stimulated AP-1 transcriptional activity (Figure 8A). The expression of inflammatory-associated target genes of AP-1; IL-6 (Figure 8B) and GM-CSF (Figure 8C) were analysed using qRT-PCR. Stimulation with PMA significantly increased mRNA expression of both genes while pre-treatment with INI-43 was able to reduce this expression which was comparable to the effect of the JNK inhibitor, SP600125, which is an upstream inhibitor of the AP-1 signaling pathway. Activation of AP-1 requires that c-JUN be phosphorylated in the nucleus by phosphorylated JNK, although nuclear import of JNK is NLS-independent and therefore not reliant on KPNB1 [41, 42]. Western blotting was used

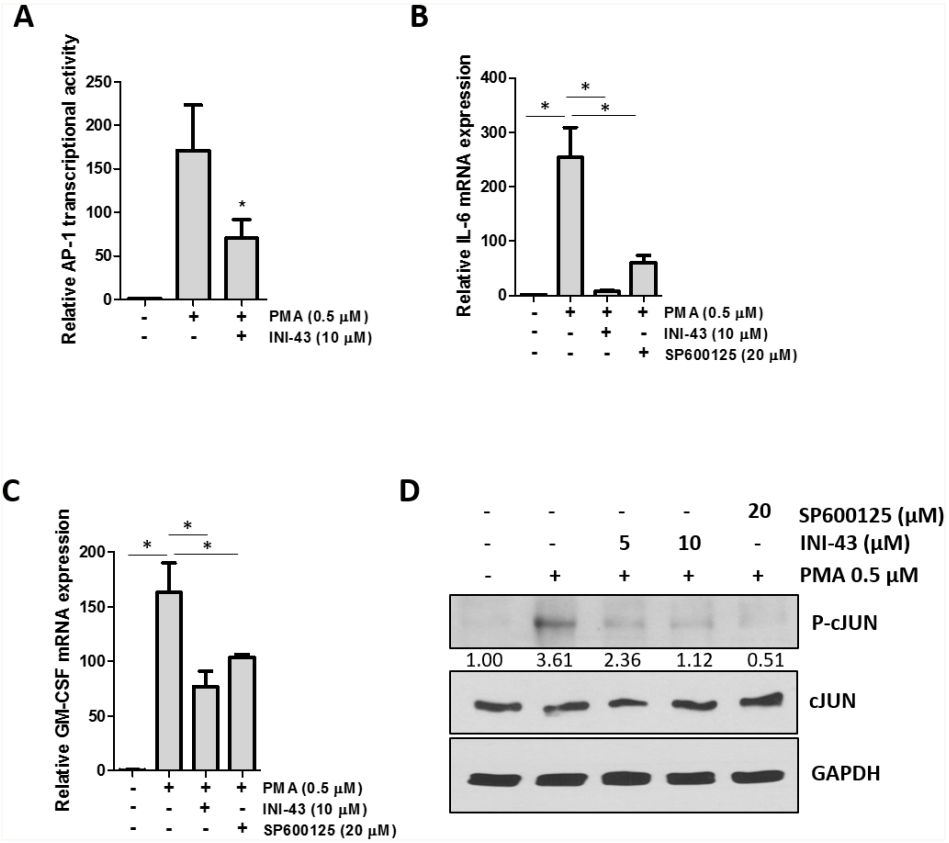

Figure 8: AP-1 activity and target gene expression in affected by KPNB1 inhibition. (A) A luciferase reporter assay showed AP-1 transcriptional activity in HeLa cells following a 3 hour 0.5 μM PMA treatment as well as a 21 hour pre-treatment with 10 μM INI-43. **(B)** qRT-PCR analysis of AP-1 target genes IL-6 and **(C)** GM-CSF shows mRNA expression following stimulation for 1 hour with 0.5 μM PMA and a 2 hour pre-treatment with 10 μM INI-43 or 1 hour pre-treatment with 20 μM SP600125. **(D)** Western blotting showed phosphorylation levels of c-JUN in HeLa cells following a 1 hour treatment with PMA and a 2 hour pre-treatment with INI-43 or 1 hour pre-treatment with SP600125. Densitometric analysis shows phosphorylated levels of c-JUN normalized to total c-JUN. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05)

to assess the phosphorylation of c-JUN and showed that PMA stimulation increased c-JUN phosphorylation while INI-43 treatment at 0.5x and 1x IC_{50} as well as SP600125 reduced phosphorylation (Figure 8D). These results show that the AP-1 transcription factor is dependent on KPNB1 for its activity and target gene expression.

Cervical cancer cell motility depends on inflammatory transcription factor activity

There is evidence in the literature that NFkB and AP-1 signaling contributes to cancer cell migration and invasion through the expression of inflammatory cytokines such as IL-6 and TNF-α [43]. We have shown above that NFkB and AP-1 signaling can be modulated through inhibiting the function of KPNB1. As a result of this we wanted to investigate whether the changes is cervical cancer cell motility seen when KPNB1 was inhibited are potentially attributed to the inhibition of NFkB and AP-1 signaling. In order to do this we performed a transwell migration assay where HeLa cells were treated with INI-43 alongside cells where NFkB was inhibited (JSH-23) as well as AP-1 signaling inhibited (SP600125). Although inhibiting NFkB signaling showed a trend towards reducing HeLa cancer cell migration the results were not significantly different from the control while inhibiting KPNB1 using INI-43 and inhibiting AP-1 signaling using SP600125 significantly reduced the migratory ability of cervical cancer cells (Figure 9A). Quantification of the migration data is shown in Figure 9B. The data suggests

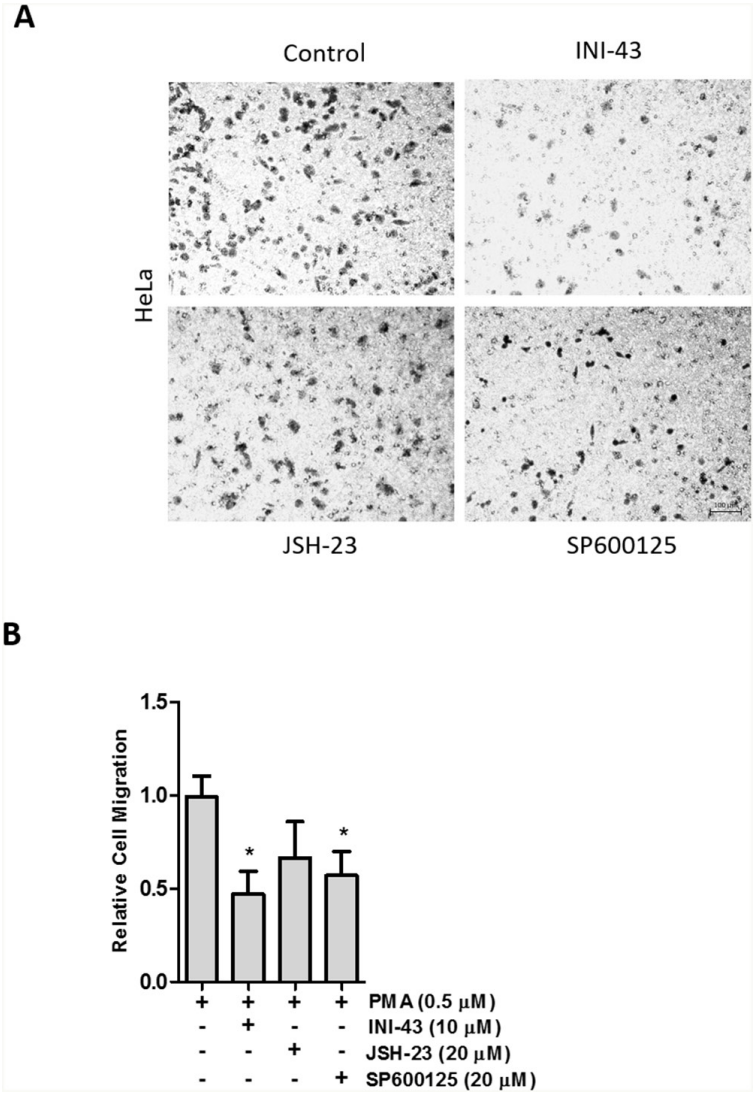

Figure 9: Cervical cancer cell migration depends on AP-1 signaling activity. (A) Representative images of a transwell migration assay showing the amount of HeLa cells that were able to migrate through the membrane in the presence of 0.5 μM PMA (control) and following a 3 hour pre-treatment with 10 μM INI-43 and 20 μM JSH-23 and a 2 hour pre-treatment with 20 μM SP600125. Scale bar represents 100 μm. **(B)** Quantification of the migration assay showing HeLa cell migration following INI-43, JSH-23 and SP600125 treatment, normalized to MTT cell viability. Results shown are the mean ± SD of experiments performed in triplicate. (*p<0.05)

that suppressed NFkB and AP-1 signaling following KPNB1 inhibition may together contribute to the inhibitory effects on cancer cell biology.

DISCUSSION

The increased expression of KPNB1 has been shown to increase nuclear import efficiency in transformed cells [44]. This enhanced access of cargo into the nucleus is suggested to sustain the high metabolic and proliferative demands of cancer cells. Cancer cells have been found to require KPNB1 for their proliferation although the exact mechanism of action for this requirement is not fully understood. It could be attributed to either; the essential role of KPNB1 in mitosis or linked to the inability of certain cargoes to gain access into the nucleus and perform their jobs in promoting proliferation [45]. This led us to question whether other cancer phenotypes are also dependent on KPNB1.

In our study we have shown that inhibiting KPNB1 reduced migration and invasion of cancer cells. As cancer cells require the activity of matrix-modifying enzymes to invade surrounding tissue, we looked at whether MMP activity was affected by KPNB1 inhibition. MMP activity was found to be significantly inhibited in cervical cancer cells following INI-43 treatment. Fan *et al.* (2016) found MMP-9 to be upregulated in cervical cancer tissue which correlated with a poor patient prognosis. Inhibiting MMP-9 activity in SiHa and HeLa cervical cancer cells reduced their migratory and invasive ability [46]. Similarly, MMP-2 expression was also found to be decreased following KPNB1 inhibition while expression of the inhibitors of matrix metalloproteases, TIMP-1 and TIMP-2, were significantly increased. TIMPs are known to be downregulated in a variety of cancer cell lines which is thought to contribute to the invasive properties of cancer cells. The overexpression of TIMP-2 in a highly metastatic melanoma cell line was able to inhibit metastasis [47, 48]. This work suggests that KPNB1 may play an important role in the migratory and invasive potential of cervical cancer cells. KPNB1 could be functioning as one of the key transporters of transcription factors associated with cancer cell migration, invasion and metastasis.

The transcription factors NFkB and AP-1 play a primary role in regulating MMP expression [14, 29, 49]. As both NFkB and AP-1 require access to the nucleus in order to be functional we hypothesized that inhibiting nuclear import via KPNB1 may lead to their inactivity and ultimately various downstream effects on cancer cell biology. We identified KPNB1 to be required for the subcellular translocation and activity of the NFkB transcription factor. A study by Liang *et al.* (2013) found KPNB1 to be responsible for the cellular translocation of NFkB p65 and inhibiting its function resulted in the retention of NFkB p65 in the cytoplasm leading to reduced NFkB transcriptional activity [23]. Our data is in support of these findings. NFkB is an important mediator of inflammation while other transcription factors such as AP-1 have also been known to initiate expression of inflammatory cytokines [26, 33]. In this study we questioned whether inhibiting NFkB and AP-1 activity via inhibiting KPNB1 associated with the inhibition of inflammatory gene expression. KPNB1 was inhibited using two approaches; RNA interference and drug-mediated inhibition. The canonical NFkB pathway as well as c-JUN activation pathway was stimulated in cell culture using PMA which enhanced IL-1β, IL-6, TNF-α and GM-CSF expression. We show that stimulated cytokine expression could be blocked following inhibition of KPNB1 or through transcription factor specific inhibitors further confirming the reliance of NFkB and AP-1 activity on the nuclear importer. The pro-inflammatory cytokines; IL-1β, IL-6, TNF-α and GM-CSF have been implicated in cell biology changes required for tumour progression [11, 50–52]. These changes include increased migratory ability and invasive potential which are required for the epithelial-to-mesenchymal transition (EMT) that precedes metastasis [53]. On activation of the transcription factor, NFkB, target genes such as TNF-α and IL-6 can subsequently form a positive feedback loop further activating NFkB as well as other transcription factors such as AP-1. Together activation of these transcription factors has been associated with down-regulated E-cadherin expression and upregulation of matrix metalloprotease production ultimately promoting EMT [29, 54, 55].

To identify whether either NFkB or AP-1 are required for the migration of cervical cancer cells, each transcription factor was individually inhibited alongside INI-43 and the effect on migration analysed. Although inhibition of the AP-1 signaling pathway was able to significantly reduce migration, neither inhibition of AP-1 or NFkB on their own were able to reduce migratory potential of cancer cells to the extent of the nuclear import inhibitor. Transcription factors of the FOS/JUN, CREB/ATF and NFkB families are known to act synergistically to significantly increase the expression of the same target gene. It has therefore been proposed that inhibiting more than one transcription factor acting synergistically may be a more appropriate approach [56]. Darnell (2002) proposed that inhibiting nuclear import proteins may be a way of specifically targeting multiple overactive transcription factors [57]. Our data provides evidence that supports Darnell's proposal, showing that inhibiting KPNB1 affects AP-1 and NFkB transcriptional activities required for cancer cell biology.

To our knowledge this study is a first to identify KPNB1 as a potential therapeutic target for inflammatory signaling in cancer associated with enhanced motility and invasiveness. Much research has been done on targeting individual transcription factors as a chemotherapeutic approach. This approach is often associated with broad-range side effects given the diverse role of each transcription

factor in normal cellular functioning. Cancer cells however, have been reported to become "addicted" and highly dependent on the activity of certain transcription factors or oncogenes, hence inhibiting their function is thought to effect cancer cells to a greater extent than non-cancer cells [58]. Ideally a targeted approach would be required to limit such off-target effects. Targeting KPNB1 as a means of transcription factor inhibition may be a favorable approach as KPNB1 is overexpressed in cervical cancer tissue in comparison to normal cervical tissue and we have previously shown that normal cells are less dependent on KPNB1 for proliferation and survival [5]. Our research group has also previously shown that inhibiting KPNB1 using INI-43 reduced tumour growth in a mouse model [37]. These results further characterize the nuclear import protein, KPNB1, as a potential anticancer target.

MATERIALS AND METHODS

Cell culture

Human cervical carcinoma cell line, HeLa and SiHa, were obtained from the American Type Culture Collection (ATCC). Cells were cultured under adherent conditions in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% Fetal Calf Serum (FCS) (HiClone, Thermo Scientific, USA). Cells were incubated at 37°C in 95% air and 5% CO_2. Cell lines were authenticated by DNA profiling using the Cell ID system (Promega, USA).

RNA interference & drug treatment

Short-interfering RNA (siRNA) was used to inhibit KPNB1 gene expression (sc-35736, Santa Cruz Biotechnology, USA). Control siRNA (SIC001, Sigma-Aldrich, USA) consisting of a scrambled RNA sequence was used as a non-silencing control. The cells were transiently transfected with 20 nM (35 mm plate)/ 52 nM (60 mm plate) siRNA using TransFectin Lipid Reagent (Bio-Rad, USA). Protein was extracted or cells assayed 30-48 hrs post transfection. The effect of KPNB1 knockdown was confirmed by Western Blot analysis. The nuclear import inhibitor, INI-43, was used at a concentration of 10 µM unless otherwise stated [37]. Other inhibitors include the NFkB inhibitor, JSH-23, used at 20 µM (Sigma-Aldrich, USA) and the JNK inhibitor, SP600125, used at 20 µM (Sigma-Aldrich, USA). The phorbol ester, phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, USA), was used to stimulate a cellular inflammatory response.

Migration and invasion assays

Cells were seeded into 35 mm dishes and treated accordingly before being trypsinized, resuspended in 0.1% FCS-containing DMEM and seeded in equal quantities into 12-well Transwell migration chambers (Greiner Bio-One, Austria) or 24-well matrigel covered Transwell invasion chambers (BD Biosciences, USA) with an 8 µm pore size. The chambers were placed into a lower chamber containing 20% FCS-containing DMEM and the cells allowed to migrate/invade through the membrane or matrigel matrix over 24 hrs. The cells that were unable to move through the membrane were removed while the remaining cells were fixed in methanol, stained with crystal violet, counted and imaged using a Zeiss Primovert inverted phase microscope. Results were normalized to an MTT cell viability assay (according to the manufacturer's protocol, Sigma-Aldrich, USA) and western blotting confirmed KPNB1 knockdown.

Gelatin zymography

Cells were seeded into 35 mm plates, treated and serum-free media placed onto the cells to condition for 16 hrs. Conditioned media was collected and centrifuged to remove any cellular debris, combined with sample buffer, loaded into a gelatin gel and run at 125 V. The gel was removed and placed in a renaturing solution before being placed in the developing buffer and allowed to incubate at 37°C overnight to facilitate gelatinase activity. The staining solution was used to dye the gel while the destaining solution exposed areas of gelatinase activity.

Immunofluorescent microscopy

Immunofluorescent analysis of NFκB p65 was performed on HeLa cells plated over glass coverslips and transfected with siRNA (48 hrs), treated with 10 µM INI-43 (3 hrs) and/or stimulated with 0.5 µM PMA for 1 hour before being fixed in 4% paraformaldehyde. Cells on the coverslips were permeabilised using 0.25% Triton X-100 in PBS for 10 mins followed by three 5 min PBS washes. Cells were blocked in 1% BSA in PBS-T + 0.3 M Glycine (for quenching) at room temperature for 30 mins. Cells were subsequently incubated with α-NFκB p65 primary antibody (1:200; sc-7151x, Santa Cruz Biotechnology, USA) in 1% BSA in PBS-T for 1 hour at room temperature, followed by three 5 min PBS washes. Cy3-conjugated goat anti-rabbit secondary antibody (1:300, Jackson ImmunoResearch, USA) in 1% BSA in PBS-T was applied for a further 1 hour. Cells were washed three times for 5 mins in PBS, nuclei stained with DAPI (100 ng/ml) and mounted onto glass slides with Mowiol. Fluorescent images were captured at 100x in oil immersion using a Zeiss Axiovert 200M fluorescent microscope with AxioVision 4.8 Zeiss software and an AxioCam HRm.

Protein harvest and western blot analysis

Following relevant treatment of cells, protein was either harvested on ice in RIPA buffer containing

a fresh mixture of complete protease inhibitors (Roche, Switzerland) and 0.1 M Sodium Orthovanadate to inhibit phosphatase activity or fractionated using the NE-PER Nuclear and Cytoplasmic Extraction Reagents according to the manufacturer's instructions (Thermo Scientific, USA). Protein concentrations were determined using the Bicinchoninic Acid (BCA) assay kit (Pierce, Thermo Scientific, USA). Western blot analysis was performed using rabbit anti-KPNB1 (H-300) (sc-11367, Santa Cruz Biotechnology, USA), rabbit anti-NFκB p65 (H-286) (sc-7151x, Santa Cruz Biotechnology, USA), rabbit anti-NFκB p50 (H-119) (sc-7178x, Santa Cruz Biotechnology, USA), rabbit anti-p-c-Jun (Ser63/73) (sc-16312-R, Santa Cruz Biotechnology, USA), rabbit anti-c-Jun (D) (sc-44, Santa Cruz Biotechnology, USA), mouse anti-GAPDH (0411) (sc-47724, Santa Cruz Biotechnology, USA), rabbit anti-β-tubulin (H-235) (sc-9104, Santa Cruz Biotechnology, USA) and rabbit anti-TBP (N-12) (sc-204, Santa Cruz Biotechnology, USA).

Electromobility shift assay (EMSA) and supershift assay

Nuclear protein was extracted from treated HeLa cells and concentrations quantified as before. The wild-type NFkB oligonucleotides; 5'-AGTTGAGGGGACTTTCCCAGGC-3' and 3'-TCA ACTCCCCTGAAAGGGTCCG-5' were labelled using the Biotin 3' End Labelling Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Equal parts of labelled probe were annealed under the following conditions; 3 mins- 95°C, 10 mins- 65°C, 60 mins- 37°C and 1 min- 25°C. The binding reaction contained; 5 µg protein, incubation buffer, poly DI/DC and 5 µl biotin-labelled double-stranded NFκB oligonucleotide. For the supershift analysis, 2 µl NFκB p65/p50 antibody was added to the binding reaction. For the controls 1 µl 50 µM unlabeled double-stranded wild-type oligonucleotide or unlabeled double-stranded mutant oligonucleotide, 5'-AGTTGAGGCGACTTTCCCAGGC-3' and 3'-GCC TGGGAAAGTCGCCTCAACT-5', was added to the binding reaction. Samples were electrophoresed on a polyacrylamide gel and detected using the Chemiluminescent Nucleic Acid Detection Module (Thermo Scientific, USA).

Luciferase reporter assays

HeLa cells were cultured in 24-well plates and transfected with 50 ng of the NFκB p65 luciferase reporter construct (containing 5 copies of the p65 binding site, Promega, USA), the AP-1 luciferase reporter construct (containing four copies of the AP-1 binding site, [28]) or the full-length IL-6 promoter construct in the pXP2 luciferase vector (Gifted by Assoc Prof Luiz Zerbini, ICGEB, Cape Town) [59] and 5 ng of the pRL-TK plasmid (encoding Renilla luciferase) to normalize for transfection efficiency. Thereafter cells were transfected with siRNA (48 hrs), treated with 10 µM INI-43 (3-24 hrs), 20 µM JSH-23 (24 hrs) and/or stimulated with 0.5 µM PMA for 1-8 hours before being lysed in 100 µl 1x Passive Lysis Buffer (Promega, USA). Luciferase firefly activity was assayed using the Dual Luciferase kit (Promega, USA) on the Glomax 96 microplate luminometer (Promega, USA). Promoter activity was normalized to Renilla luciferase activity.

Quantitative real time PCR

Quantitative real-time PCR was performed using the StepOne Real-time PCR system (Applied Biosystems, USA). RNA was isolated from treated cells using Qiazol (Qiagen, Netherlands), according to the manufacturer's instructions. Synthesis of complementary DNA used 2 µg RNA and 2-4 µl of this was amplified using the KAPA SYBR Fast mastermix (KAPA Biosystems, South Africa) for qRT-PCR analysis. The sequence of qRT-PCR primers were as follows; MMP-2 F: 5' TGGCGATGGATACCCCTTT 3', R: 5' TTCTCCCAAGGTCCATAGCTCAT 3', TIMP-1 F: 5' AGAGACACCAGAGAACCCA 3', R: 5' TGATGACGAGGTCGGAATTG 3', TIMP-2 F: 5' CATGATCCCGTGCTACATCTC 3', R: 5' TTGATGC AGGCGAAGAACT 3', IL-1β F: 5' CCACCTCCAGG GACAGGATA 3' and R: 5' TGGGATCTACACTCTC CAGC 3', IL-6 F: 5' GGATTCAATGAGGAGACTTGCC 3' and R: 5' CAGGCTGGCATTTGTGGTTG 3', TNF-α F: 5' GTAGCCCATGTTGTAGCAAACC 3' and R: 5' TGA TGGCAGAGAGGAGGTTG 3', GM-CSF F: 5' GAC ACTGCTGCTGAGATGAATG 3' and R: CAGTGC TGCTTGTAGTGGCT 3', GAPDH F: 5'GGCTCT CCAGAACATCATCC 3' and R: 5' GCCTGCTTC ACCACCTTC 3'. Samples were standardized to the house-keeping gene, GAPDH. Analysis was carried out using the comparative threshold cycle (C_T) method.

Statistical analysis

Experiments were performed in triplicate and represented as mean ± SD and repeated at least two independent times unless stated otherwise. The student's *t* test was performed in GraphPad Prism V5.0 for all comparisons. A *p* value of $p<0.05$ was considered statistically significant.

Abbreviations

KPNB1- Karyopherin beta 1, NLS- Nuclear localisation sequence, NPC- Nuclear pore complex, NFkB- Nuclear factor kappa B, AP-1- Activator protein 1, IL-1β- Interleukin 1 beta, IL-6- Interleukin 6, TNF-α- Tumour necrosis factor alpha, MMP- matrix metalloprotease,

TIMP- Tissue inhibitor of metalloproteinase, GM-CSF- Granulocyte macrophage colony stimulating factor, JNK- cJUN N-terminal kinase

Author contributions

Tamara Stelma contributed to the development of methodology, acquisition and analysis of data and the writing of the manuscript.

Virna D. Leaner contributed to the concept and design of the study, review of the manuscript and study supervision.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

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Critical Review

Targeting Nuclear Transporters in Cancer: Diagnostic, Prognostic and Therapeutic Potential

Tamara Stelma[1]
Alicia Chi[1]
Pauline J. van der Watt[1]
Annalisa Verrico[2]
Patrizia Lavia[2]
Virna D. Leaner[1]*

[1]*Division of Medical Biochemistry, Department of Integrative Biomedical Sciences, SAMRC/UCT Gynaecological Cancer Research Centre, Institute of Infectious Disease and Molecular Medicine, Faculty of Health Sciences, University of Cape Town, Cape Town, South Africa*
[2]*Institute of Molecular Biology and Pathology, National Research Council of Italy, C/O University of Roma "La Sapienza", Rome, Italy*

Abstract

The Karyopherin superfamily is a major class of soluble transport receptors consisting of both import and export proteins. The trafficking of proteins involved in transcription, cell signalling and cell cycle regulation among other functions across the nuclear membrane is essential for normal cellular functioning. However, in cancer cells, the altered expression or localization of nuclear transporters as well as the disruption of endogenous nuclear transport inhibitors are some ways in which the Karyopherin proteins are dysregulated. The value of nuclear transporters in the diagnosis, prognosis and treatment of cancer is currently being elucidated with recent studies highlighting their potential as biomarkers and therapeutic targets. © 2016 IUBMB Life, 68(4):268–280, 2016

Keywords: *Karyopherin/Importin; CRM1/Exportin; nuclear transport; cancer; diagnostic/prognostic cancer biomarkers; therapeutic target*

Introduction

Protein trafficking is fundamental to cellular function and cell survival. Of the protein trafficking systems found in cells, access into the nucleus through the nuclear pore complex (NPC) is essential for the functioning of many proteins. Proteins smaller than 20–40 kDa can passively diffuse through the NPC while larger proteins require active facilitated transport to gain access in or out of the nucleus.

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**Address correspondence to: Virna Leaner, Division of Medical Biochemistry, Faculty of Health Sciences, University of Cape Town, Observatory, Cape Town 7925, South Africa. Tel: +27-21-406-6250. Fax: +27-21-406-6061*
E-mail: virna.leaner@uct.ac.za
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Facilitated transport of numerous proteins is achieved via the action of the Karyopherin proteins. The Karyopherin superfamily consists of both the Karyopherin beta/Importin beta family and the Karyopherin alpha/Importin alpha family of adaptor proteins. The Karyopherin beta family forms the major class of soluble transport receptors. Its members interact with and import and/or export cargo proteins and certain RNAs across the nuclear envelope. Thus far there have been 20 genes encoding Karyopherin beta family members identified in the human genome including 10 proteins involved in nuclear import, seven proteins involved in nuclear export, two bidirectional transporters and one transporter that remains uncharacterised (Table 1) (40). The Karyopherin alpha family of adaptor proteins bind the nuclear localisation signal (NLS) of cargo proteins and link them to the Karyopherin beta transport protein, hence playing the role of an adaptor. Seven Karyopherin alpha isoforms are currently characterised, which are grouped into three subfamilies: A1, A2 and A3. The A1 subfamily comprises Karyopherin A2 and Karyopherin A7, the A2 subfamily includes Karyopherin A3 and Karyopherin A4 and, finally, the A3 subfamily consists of

TABLE 1 ***Nuclear transporters associated with cancer***

	Symbol	*Synonyms*	*Cargoes*	*Cancer*
(A) Import				
Karyopherin beta 1	KPNB1	NTF97, IPOB, MGC2155, MGC2156, MGC2157, IMB1, Impnb, IPO1	NFκB, NFAT, CREB, PTHrP, PRPF31, SRY/SOX-9, Cyclin B1 (1), SREPB2 (2)	Cervical (3), gastric (4), breast (5)
Transportin 1	TNPO1	KPNB2, MIP, TRN, IPO2, MIP1	HPV16E6 oncoprotein (1)	–
Transportin 2	TNPO2	IPO3, KPNB2B, FLJ12155, TRN2	c-Jun, ribosomal proteins (1)	–
Transportin 3	TNPO3	LGMD1F, TRN-SR, MTR10A, TRN-SR2, IPO12	SRSF1 (6)	–
Importin 4	IPO4	Imp4, FLJ23338	Vitamin D receptor, HIF1-α (1)	–
Importin 5	IPO5	KPNB3, RANBP5, IMB3, MGC2068, Pse1	c-Jun (1)	–
Importin 7	IPO7	RANBP7, Imp7	c-Jun, Histone H1, CREB, SMAD3, glucocorticoid receptor (1)	–
Importin 8	IPO8	RANBP8, IMP8	SMADS, signal recognition particle protein 19 (1)	–
Importin 9	IPO9	Imp9, FLJ10402	c-Jun, PP2A (1)	–
Importin 11	IPO11	RanBP11	L12, UbcM2 (1)	–
Karyopherin alpha 1	KPNA1	SRP1, RCH2, NPI-1, IPOA5	STAT1, STAT2, STAT3 (1)	–
Karyopherin alpha 2	KPNA2	RCH1, SRP1alpha, IPOA1, QIP2	Type 1 parathyroid hormone receptor (1)	Cervical (*7*), breast (*8*), prostate (*9*), brain (*10*), gastric (*11*), ovarian (*12*), bladder (*13*), urothelial (*14*), liver (*15*), melanoma (*16*), lung (*17*), oesophageal (*18*)
Karyopherin alpha 3	KPNA3	SRP1gamma, SRP4, hSRP1, IPOA4	NFκB p50/p65 (*1*)	–
Karyopherin alpha 4	KPNA4	QIP1, SRP3, IPOA3, MGC12217, MGC26703	NFκB p50/p65 (*1*)	–
Karyopherin alpha 5	KPNA5	SRP6, IPOA6		–

TABLE 1 *(Continued)*

	Symbol	*Synonyms*	*Cargoes*	*Cancer*
(A) Import				
Karyopherin alpha 6	KPNA6	IPOA7, KPNA7, MGC17918, FLJ11249	HNRNPU, LMNB1, HNRNPL (*19*)	–
Karyopherin alpha 7	KPNA7	IPOA2	GATA-3 (*1*)	Pancreatic (*20*)
(B) Export				
Exportin 1	XPO1	CRM1, emb	Cyclin D1, IκB, NFAT (*1*), [full list (*21*)]	Cervical (*3*), gastric (22), ovarian (*23*), pancreatic (*24*), osteosarcoma (*25*), brain (*10*), acute myeloid leukaemia (26), oesophageal (27)
CSE1 chromosome segregation 1-like (yeast)	CSE1L	CAS, XPO2, CSE1	Karyopherin alpha proteins (28)	Bladder (*29*, (30)), leukaemia (*28*), breast (*31*), liver (*32*), colon (*33*), ovarian (*34*), non-Hodgkin's lymphoma (*35*)
Exportin 5	XPO5	KIAA1291	Staufen2 (1), pre-miRNA (36)	Colorectal (36)
Exportin 6	XPO6	RANBP20, KIAA0370, FLJ22519	Actin (1)	–
Exportin 7	XPO7	RANBP16, KIAA0745	P50RhoGAP (1)	–
Exportin t	XPOT	XPO3	tRNA (1)	–
Ran binding protein 17	RANBP17			–
(C) Bidirectional				
Exportin 4	XPO4	FLJ13046, KIAA1721	SRY/SOX-2 (*37*), SMAD3 (38)	Liver (39)
Importin 13	IPO13	IMP13, KIAA0724, RANBP13	c-Jun, glucocorticoid receptor (1)	–
(D) Uncharacterised				
Ran binding protein 6	RANBP6			–

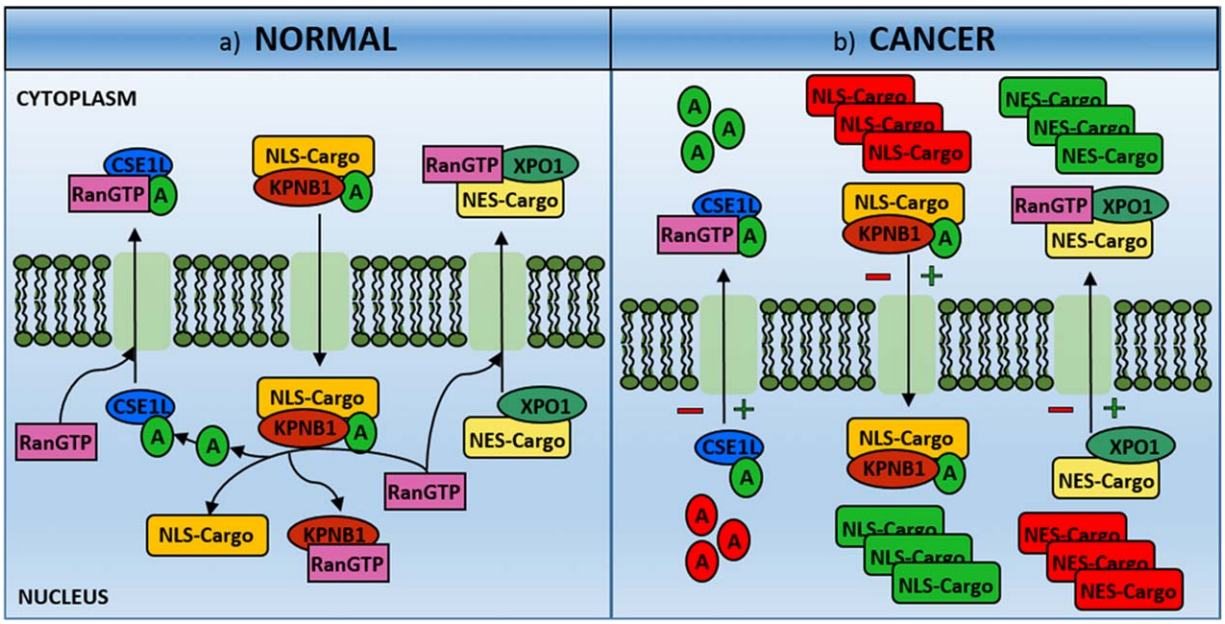

FIG 1

Proposed dysregulation of nuclear–cytoplasmic transport of cargo proteins in cancer cells in comparison to normal. (a) In normal cells protein cargo containing a nuclear localisation signal (NLS-cargo) is recognised and bound by a Karyopherin alpha (A) transport protein after which this complex is bound by Karyopherin beta 1 (KPNB1) and allowed passage through the nuclear pore complex (NPC). Upon association with RanGTP the cargo protein dissociates and the nuclear transport proteins are recycled back into the cytoplasm. Recycling of the Karyopherin alpha protein occurs through binding to CSE1L and in association with RanGTP is transported back through the NPC into the cytoplasm. Nuclear protein cargo containing a nuclear export signal (NES-Cargo) is recognised and bound by XPO1 where again in association with RanGTP is transported through the NPC into the cytoplasm. (b) In cancer cells it is proposed that the overexpression of nuclear transporters (green +), including KPNB1, XPO1 and CSE1L leads to an increased rate of transport through the NPC. As a result their respective cargoes potentially accumulate in the cytoplasm or nucleus. Nuclear transporters can also be mislocalized (red −) rendering them nonfunctional and inhibiting transport through the NPC which causes accumulation of cargo proteins in their compartment of origin. Both overexpression of nuclear transporters and their mislocalization have been seen in cancer and associated with increased proliferation and increased survival.

Karyopherin A1, Karyopherin A5 and Karyopherin A6 (Table 1) (41).

The classical nuclear import pathway involves a Karyopherin alpha isoform recognising and binding a cargo containing a classical nuclear localisation signal (cNLS), which is then bound by Karyopherin beta 1 (KPNB1). This trimeric complex is then shuttled through the NPC. An alternative path of nuclear import involves the cargo protein bypassing the need for an adaptor and binding KPNB1 directly. Cargo proteins such as parathyroid hormone-related protein (PTHrP) and sterol regulatory-related protein (SREPB2) use this mode of nuclear import (2). Passage through the NPC is mediated by the interactions between Phe-Gly repeat-containing nucleoporins of the NPC and KPNB1. Upon arrival in the nucleus, a key event is the binding of RanGTP to the KPN/cargo complex, which promotes dissociation of the import complex, allowing the cargo to remain in the nucleus and the transport proteins to be recycled (42,43). The nuclear export protein, CSE1L, is responsible for recycling the Karyopherin alphas back into the cytoplasm. XPO1, the most well-characterised export protein, binds cargoes containing a leucine-rich nuclear export signal (NES). RanGTP stabilizes the XPO1-bound NES–cargo complex that can then pass through the NPC towards the cytoplasm. When in the cytoplasm, the hydrolysis of RanGTP to RanGDP allows the dissociation of the export protein and the cargo (Fig. 1) (44).

Although the best described role of the Karyopherin proteins is the nuclear import and export of cargo proteins, they also play an important role in mitosis and other cell cycle-regulated functions including regulating the assembly of the NPC and nuclear membrane at mitotic exit as well as DNA replication in S-phase (45,46). During normal cellular homeostasis, the nuclear transport system is regulated through various mechanisms including signal transduction, cell cycle, immune response, development and stress (42). Dysregulation of nuclear transport has been associated with carcinogenesis and poor prognosis in a multitude of cancers. This review aims to discuss recent work revolving around the role of key nuclear transport receptors in cancer. It will cover the dysregulation of nuclear transport pathways and focus on the potential of targeting these transporters in the diagnosis, prognosis and treatment of cancer.

Dysregulation of Nuclear Transport in Cancer

The Karyopherin superfamily of proteins play a fundamental and indispensable role in normal cell physiology by providing the means of trafficking between the cytoplasm and the nucleus as well as in other cellular processes such as mitosis. As intracellular localization of many proteins as well as the nuclear transporters themselves has an impact on their activity status and/or function, the correct spatial arrangement of proteins at the correct time is critical. It is therefore no surprise that impaired regulation of nuclear–cytoplasmic transport associates with pathogenesis. A well-researched area is the association of nuclear transport dysregulation and cancer, which most commonly leads to the mislocalization of cancer-associated cargo proteins (Fig. 1). For example, the nuclear accumulation of the DNA repair protein, NBS1, has been shown to correlate with gastric cancer progression, while the cytoplasmic accumulation of Wilms' tumour protein, Wt1, was observed more frequently in malignant tissues than normal (11,47). There are many junctions in the nuclear transport pathways at which dysregulation can occur; here we discuss mechanisms that are more frequently observed and are known to be linked to cancer. These include: (i) the altered expression of the nuclear transporters, (ii) altered localization of nuclear transporters, (iii) the disruption of endogenous nuclear transport inhibitors and (iv) the mechanistic implications of nuclear transporters in mitotic division and genetic instability.

Altered Expression of the Nuclear Transporters

The elevated expression of the Karyopherin proteins associates with the global dysregulation of protein transport and this has been observed in various types of cancer. Amongst all members of the Karyopherin family, XPO1, KPNB1, KPNA2 and CSE1L are the most frequently reported to be overexpressed in cancer (Table 1). Kuusisto et al. found that the increased expression of these Karyopherin proteins in transformed cells correlates with enhanced nuclear import and export efficiencies in transformed cells (48). The increased expression and thus transportation across the nuclear membrane is possibly a mechanism devised by cancer cells to cope with the increased metabolic and proliferative demands. The increased expression of import proteins, in particular, might allow for increased nuclear entry of proteins that have oncogenic tumour-promoting functions, for example, ERK1/2, c-Myc and E2F1 (49–51).

Whilst the link between Karyopherin overexpression and cancer has been the subject of numerous studies, few researchers have addressed the underlying mechanism leading to their overexpression. Using molecular and bioinformatics approaches, van der Watt et al. showed that the overexpression of both KPNB1 and KPNA2 is primarily due to dysregulated E2F/Rb activity in cancer cells. The constitutive activation of E2F in, *e.g.*, cervical cancer cells was found to associate with increased expression of both KPNB1 and KPNA2 (7). It is well known that HPV infection in cervical cancer results in E2F dysregulation and thus elevated KPNB1 and KPNA2 in this cancer. The E2F/Rb pathway is disrupted in a remarkably high proportion of human cancers through other mechanisms leading to the same overexpression phenotype (52). Interestingly, Kuusisto et al. showed that the extent of overexpression of KPNB1 correlates with disease state in the MCF10 human breast tumour progression system, suggesting that its overexpression not only correlates with E2F dysregulation but can vary according to tumour progression state (5). Other mechanisms for altered Karyopherin expression have been reported. A recent study showed elevated KPNA2 expression resulting from decreased expression of microRNA-26b in epithelial ovarian carcinoma. MicroRNA-26b directly targets KPNA2 by repressing its translation and thus reduced microRNA-26b resulted in elevated KPNA2 expression (53).

Quan et al. used bioinformatic approaches to predict transcription factors that bind the promoter regions of Karyopherin beta genes and identified Sp1, NRF-2, HEN-1, RREB-1 and NFY as potential regulators of Karyopherin beta expression (54). In line with this, van der Watt et al. found that increased NFY and Sp1 expression in cervical cancer and transformed are both potential contributors to XPO1 overexpression in these cells (7). Interestingly, Kahle et al. found that NFY-A is imported into the nucleus by KPNB1 where it acts to transcribe its downstream targets (55). This finding could explain why the elevated expression of KPNB1 has been observed concomitant with elevated XPO1 in cervical and gastric cancer (*3*, *4*, *22*). van der Watt et al. also showed that under DNA damage conditions the elevated p53 level plays a repressive role in the transcription of the *xpo1* gene (3). It is not surprising then that XPO1 overexpression is commonly observed in cancer as p53 is often expressed at low levels or contains mutations in many cancers (56). Interestingly, XPO1 has also been shown to be involved in p53 nuclear export (3), suggesting that a possible feedback regulatory mechanism may exist. Under DNA damage conditions p53 expression increases and enters the nucleus, thereby repressing XPO1 expression. The reduced XPO1 expression will in turn enhance p53 retention in the nucleus further repressing XPO1 expression. In p53-deficient or -mutant cells the repression of XPO1 expression is relieved, likely leading to XPO1 overexpression.

The CSE1L protein, also a member of the Karyopherin family involved in recycling the KPNA adaptors into the cytoplasm, is another member commonly reported to be overexpressed in cancer including bladder, leukaemia, breast, colon, ovarian and non-Hodgkin's lymphoma (*28*, *31*, *34*, *35*). Its overexpression has been attributed to gene amplification (31). More recent work by Winkler et al. demonstrated a repressive role of p53 in CSE1L and KPNA2 transcription in hepatocellular carcinoma mediated through p21 (57). As p53 loss-of-function is a frequent occurrence in cancer it is not surprising to observe overexpression of CSE1L in cancer.

The altered expression of nuclear transporters associated with cancer more often involves elevated expression; however,

downregulation of nuclear transporters has also been linked to cancer. Exportin 4 (XPO4) is an example although the exact underlying mechanism driving its downregulation is still unknown (39).

Altered Localization of Nuclear Transporters

The ability of nuclear transporters to efficiently execute their functions depends on their ability to interact correctly with other members of the transport cycle. A truncated KPNA lacking part of the cargo-NLS-binding domain has also shown failure to interact with and transport p53 into the nucleus in breast cancer cells. Despite the KPNB1-binding domain remaining intact in the mutant KPNA, it was localized predominantly in the cytoplasm and perinuclear region as opposed to the even distribution observed in wild-type KPNA (58). This suggests that cargo recognition and binding by KPNA is a prerequisite for nuclear entry, and that pairing with KPNB1 alone is not sufficient. A similar observation was made by Melo et al., where an inactivating mutation in Exportin 5 (XPO5) found in a subset of human tumours rendered it unable to export itself as well as its precursor microRNA cargoes out of the nucleus (36). As a result, the pre-microRNA processing efficiency was decreased leading to defects in microRNA production. Ultimately, failure to generate mature microRNA leads to the impairment of the post-translational regulation of target genes and altered expression profile.

Post-translational modification can also regulate protein localization, which is well demonstrated by the CSE1L protein. CSE1L is involved in more than one function inside the cell, but its role in nuclear transport is specifically carried out when localized to the nucleus (59). Its localization is phosphorylation-dependent, while AKT-mediated phosphorylation is required for its nuclear localization in ovarian cancer; MEK-1-mediated phosphorylation retains it in the cytoplasm (60,61). Furthermore, siRNA-mediated inhibition of CSE1L expression in ovarian cancer cells which exhibited predominant nuclear localization of CSE1L showed increased sensitivity to cisplatin treatment compared to breast and colon cancers which showed predominant cytoplasmic localization (60). This suggests that the nuclear CSE1L plays an oncogenic role and could contribute to the resistance against cytotoxic agents. The aberrant function of the upstream kinases can thus result in the incorrect localization of CSE1L and resultant pathogenesis.

Disruption of Endogenous Nuclear Transport Inhibitors

Cells also use endogenous nuclear transport inhibitors to regulate nuclear trafficking. The two most well-known endogenous inhibitors are Complement Component 3 (CC3/TIP30) and Aplasia Ras Homolog Member 1 (ARH1/NOEY2). Both proteins were found to have reduced expression or absence in a wide range of cancers. Knockdown studies concluded that these proteins function to suppress the cancer phenotype, while restoration of their expression was unfavourable to cancer development. Their ability to negatively regulate nuclear transport was only recognised about a decade later (62,63). Both proteins have been reported to interfere with nuclear import by physically interacting with members of the Karyopherin family. Binding of CC3 to the Karyopherins was RanGTP-independent and also associated with nucleoporins *in vivo*. Alternatively, ARH1 is thought to interact with Karyopherins through the NLS domain, thereby preventing NLS-cargo binding, and also competed with the binding of Karyopherin to Ran (64). Both inhibitors have the ability to prevent nuclear translocation of NLS-cargoes while CC3 was also able to prevent transport of M9 signal-bearing cargoes (62).The alleviation on nuclear transport and the subsequent altered cellular localization of cargo proteins has several advantages in tumour development and survival.

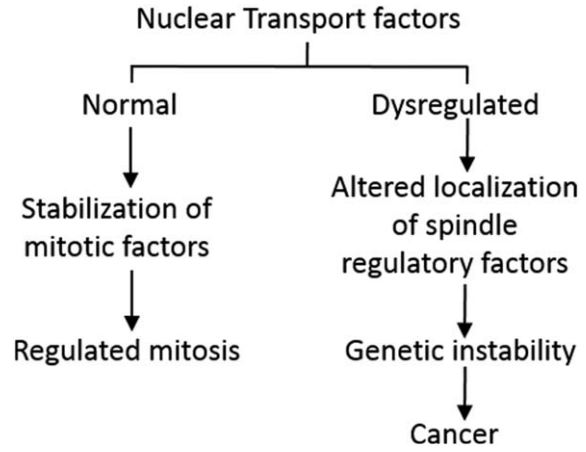

FIG 2 *Role and implications of nuclear transporters in mitosis. Under normal conditions upon cessation of nuclear transport, nuclear transport factors take on new roles in mitosis. They contribute to the stabilization of mitotic factors and regulated mitotic division. Dysregulation of the expression of nuclear transport factors has been shown to contribute to the altered localization of key spindle regulatory factors that associate with the onset of genetic instability, which is considered one of the hallmarks of cancer.*

Mechanistic Implications of Nuclear Transporters in Mitotic Division and Genetic Instability

When nucleo-cytoplasmic transport ceases, virtually all transport factors take on new roles in mitosis, acting in the functional organisation of the mitotic spindle and in the reconstitution of the interphase nucleus at mitotic exit. Modelling studies have indicated that subtle dysregulation of the expression of transport factors readily affects mitotic division and can cause significant abnormalities in chromosome segregation, whereas nucleo-cytoplasmic transport is a more robust process (65,66). Dysregulation of nuclear transport factors can therefore cause the onset of genetic instability, a cancer hallmark (Fig. 2).

The role of KPNB1 as a regulator of spindle formation and function is well described. KPNB1 generally acts by preventing the premature localisation or activity of spindle regulatory factors (67). Factors, with spindle assembly functions, regulated by KPNB1 include: the spindle pole-organizing protein NuMA

(68), the microtubule-regulatory protein HURP (69), Rae1/Nup98 (70), a regulator of the Aurora-A kinase maskin (71) and APC (72), among others. KPNB1's physical association and regulation of the *Adenomatous polyposis coli* (*Apc*) oncogene, itself a microtubule-promoting and mitotic spindle-associated factor, may be of particular relevance to the onset of colon cancer (72). A novel function through which KPNB1 can globally regulate the mitotic apparatus has recently emerged and concerns its ability to regulate, in concert with the APC/C ubiquitin ligase, the stability of mitotic spindle regulatory factors (73).

Transportin 1 (TNPO1) has documented effects on the mitotic apparatus and on post-mitotic reorganization of the interphase nucleus, a critical process that is evidently crucial for resuming transcription and replication via the cell cycle (74,75). Disruption of this process by altered TNPO1 function will also affect the genetic identity of the newly formed daughter cells after mitosis.

The CSE1L protein has predominant roles in the regulation of apoptosis, as recalled above, most probably mediated by the mislocalization of Karyopherin alpha members. It also has the ability to associate with the mitotic apparatus; altered activity of CSE1L can confer resistance to taxol but not to other chemotherapeutic drugs (76). This effect is mediated through Mek proteins, and it is particularly interesting in the light of designing new potential therapeutics (76,77).

Karyopherin alpha family members, although difficult to discuss collectively, share a common functional feature that their downregulation induces substantial levels of apoptosis (78). They contribute to mitotic control by interacting with key spindle regulatory factors such as TPX2 (79). In addition, karyopherin alpha 3 has a global role in mitosis. It interacts specifically with RCC1, the guanine exchange factor for Ran, and this interaction is inhibited by mitotic phosphorylation (78,80,81). This inhibition results in an increased concentration of free RCC1 and hence increased RanGTP production in mitosis.

The nuclear export protein, XPO1, has well-defined mitotic effects exerted at various levels of mitosis. These include the mitotic spindle (82); kinetochores, stabilizing microtubule/kinetochore interactions essential for chromosome segregation (83) and centrosomes, preventing chromosome reduplication that constitutes a major threat to genome stability (84,85). These effects are mediated through several mitotic targets that contain NES sequences. The interplay between XPO1 and mitotic cyclin B is interesting to note. XPO1 contributes to cyclin B localization and in turn cyclin B phosphorylates XPO1 to finely regulate its mitotic functions (86). It has also been reported that XPO1's interaction with survivin is important to target the chromosomal passenger protein to kinetochores (87).

Nuclear transport plays a critical role in the functioning of many cellular processes. Because of this, cells have developed elegant systems to tightly control the nuclear transport processes. Unfortunately, within each junction in the regulatory pathway lies an opportunity for errors to occur, and indeed many of these alterations have been associated with cancer.

Role of Nuclear Transporters in the Diagnosis of Cancer

Late diagnosis of cancer is a major contributing factor to poor patient outcome. Often, by the time patients present at clinics, the cancer has already progressed to later stages and the chances of the primary tumour having already metastasised are relatively high. This highlights a need for effective tools for the early detection of cancer. The ideal biomarker would be unique to cancer and present in easily obtainable patient samples such as serum or urine. Nuclear transporters have already been shown to be upregulated at the protein level in many cancer tissues but few studies recognise these proteins as potential diagnostic biomarkers. Wang et al. investigated potential biomarker targets that were both significantly upregulated in lung cancer tissues and secreted/released from lung cancer cells. They achieved this by integrating two lung adenocarcinoma cell line secretome datasets with one adenocarcinoma microarray dataset. Using this strategy they identified KPNA2 as a potential diagnostic biomarker for adenocarcinoma. Elevated KPNA2 serum levels were confirmed in a cohort of non-small-cell lung carcinoma patients ($n = 126$) in comparison to healthy individuals ($n = 64$) (17). Similar to what was observed in lung cancer patients, KPNA2 levels in serum were also significantly upregulated in oesophageal squamous cell carcinoma patients ($n = 86$) versus healthy controls ($n = 60$) validating the potential of KPNA2 as a diagnostic biomarker (88). KPNA2 has also been identified as a potential diagnostic biomarker that can differentiate between grades of astrocytoma. This, however, still requires immunohistochemical validation. The conventional criteria for differentiating between WHO grade II and III astrocytomas is particularly difficult to define. Gousias et al. found that patients diagnosed with WHO grade II astrocytoma showed little/no nuclear KPNA2 immunostaining, whereas half of the patients diagnosed with anaplastic astrocytoma (WHO grade III) showed $\geq$5% KPNA2 staining in the nucleus (89).

The nuclear transporter, CSE1L, has also shown diagnostic potential in bladder cancer and metastatic colorectal cancer (*29*, *33*). It is upregulated in bladder cancer and has been found to be secreted into the urine of these patients while it is not detected in the urine of healthy individuals (29). CSE1L has also been found to be secreted into the sera of patients with metastatic colorectal cancer (33). Thus far, no data have been published regarding the serum levels of other nuclear transport proteins in cancer. As previously mentioned, other members of the Karyopherin superfamily such as KPNB1 and XPO1 have also been shown to be upregulated in various cancers. It is thus possible that these proteins might also be secreted from cancer cells and have potential as diagnostic markers.

Nuclear Transporters as Prognostic Markers

A study by van der Watt et al. found that KPNB1, KPNA2 and XPO1 are all upregulated in cervical cancer patient samples in comparison to normal tissue. KPNB1 and XPO1 but not KPNA2 were found to be essential for the survival of cervical cancer cells. The nuclear importer, KPNB1, and exporter, XPO1, both show promise as a cancer biomarker but have not as yet been correlated to patient prognosis in cervical cancer (3). More recently, Zhu et al. found that KPNB1 was also upregulated in gastric cancer patient tissue and cells in comparison to their normal counterparts. Interestingly, Zhou et al. found XPO1 protein levels to be significantly upregulated in gastric cancer patient tissues as well, indicating that both import and export machinery is upregulated in gastric cancer. The upregulation of KPNB1 correlated positively with Ki-67 immunostaining, infiltration depth and tumour grade but not TNM stage and lymph node metastasis. On the other hand, overexpression of XPO1 positively correlated with TNM stage as well as metastasis. Both increased KPNB1 and XPO1 expression have been identified as independent prognostic factors to predict gastric cancer patient survival (*4*, *22*).

XPO1 has also been identified as a suitable independent prognostic marker in ovarian cancer, pancreatic cancer, osteosarcoma, brain cancer and acute myeloid leukaemia (*10*, *23–26*, *90*). Studies on pancreatic cancer as well as osteosarcoma revealed XPO1 overexpression to be associated with increased tumour size as well as histological grade in osteosarcoma but not pancreatic cancer. For both pancreatic cancer and osteosarcoma there is evidence suggesting that increased XPO1 expression is an indicator of reduced overall and progression-free survival (24,25). In contrast, decreased expression of the nuclear export protein, XPO4, was associated with poor overall survival in patients with liver cancer suggesting XPO4 has tumour suppressor properties (39,91). Although expression levels of nuclear transporters often enhances their prognostic value, in some cases so does their localisation within the cell. While the localisation of XPO1 between the nucleus and the cytoplasm showed no significant differences among gastric cancer samples, the cellular localisation of XPO1 in ovarian cancer has been correlated with different aspects of cancer progression (22). Enhanced cytoplasmic XPO1 has been associated with advanced ovarian tumour stage, poor differentiation and higher mitotic rate. Nuclear XPO1 levels have been associated with enhanced Cox-2 expression leading to poor overall patient survival (23). Nuclear XPO1 has also been correlated with pathological stage in gliomas (90). XPO1 localisation is also altered during oesophageal tumourigenesis, where it shifts from predominantly nuclear in normal tissue to nuclear and cytoplasmic in cancer tissue. This appears to occur in the early stages of disease progression (27).

The nuclear export protein, CSE1L, has been reported to have prognostic potential in bladder, liver and ovarian cancers (*30*, *32*, (34)). Chang et al. found urothelial carcinoma patient samples to have differing cytoplasm and nuclear CSE1L staining. While cytoplasmic CSE1L staining showed no correlation to clinical manifestations, nuclear CSE1L closely correlated with poor overall survival (30). In liver cancer, CSE1L was found to be upregulated particularly in the cytoplasm of cells in comparison to normal tissue, but the association to patient outcome was not further investigated (32).

The value of nuclear transporters such as KPNB1, XPO1 and CSE1L as prognostic tools is still under investigation and data supporting this are only available for a select few cancers. In contrast, KPNA2 has been well established as a prognostic marker in various cancers including: breast cancer, brain cancer, gastric cancer, prostate cancer, ovarian cancer, bladder cancer, liver cancer, melanoma, lung cancer and oesophageal cancer (8–11,14,15,92,93). Dahl et al.'s research in breast cancer was the first to identify KPNA2 as a prognostic marker (94). Since then high nuclear KPNA2 expression has been linked to poor patient outcome. Numerous studies have shown that KPNA2 expression correlates with overall and progression-free survival in patients and established KPNA2 as an independent prognostic marker when compared to other clinical data (*10*, (11,13–15,89,93,95,96)). Interestingly, KPNA2 expression also predicted the chances of metastasis as well as histological grade and clinical stage of tumours. High KPNA2 expression in non-invasive bladder cancer increased the risk of progression to a more invasive form (13).

Some malignancies have a high recurrence rate following therapeutic or surgical intervention and it is important to identify risk factors in these patients that might predict recurrence. KPNA2 expression has been identified as a marker of both early and more frequent recurrence in liver cancer while it is also a marker of PSA recurrence in prostate cancer (9,97,98). In meningioma, elevated KPNA2 and XPO1 expression was observed in recurrent tumours in comparison to primary tumours and this correlated with increased recurrence rates (10).

The evidence presented here highlights that KPNA2 serves as a valuable prognostic marker throughout the progression of cancer from early-stage recurrence to chances of metastasis, as well as overall survival. While there is evidence that KPNB1, XPO1, CSE1L and XPO4 have shown potential as prognostic markers, this requires further investigation and validation. Evidence from the study by Gousias et al., which shows KPNA2 expression levels correlating with XPO1 expression levels in meningioma, suggests that other nuclear transporters may too have prognostic value in cancer (10).

Targeting Nuclear Transporters as Cancer Therapeutics

The upregulation of nuclear transporters and their association with poor prognosis in cancer highlights their potential as therapeutic targets. Challenges may arise though when

TABLE 2 *Nuclear transport inhibitors*[a]

Nuclear transporters	*Inhibitor*	*Nature of the compound*	*Experimental status*
KPNB1	cSN50.1 (103)	Peptide	Cell culture
KPNA/B1	Ivermectin (104)	Antibiotic	
	cSN50.1 (103)	Peptide	Cell culture
	Bimax1 (105)	Peptide	Cell culture
	Bimax2 (105)	Peptide	Cell culture
	Karystatin 1A (106)	Small molecule	Cell culture
	Importazole (107)	Small molecule	Cell culture
TNPO1	M9M (108)	Peptide	*Xenopus* model
XPO1	Leptomycin B (109)	Antibiotic	Phase I clinical trials (discontinued)
	Ratjadone analogs (21)	Antibiotic	Cell culture
	Anguinomycin (21)	Antibiotic	Cell culture
	Goniothalamin (21)	Organic molecule	Cell culture
	Kos-2464 (21)	Small molecule	Xenograft mouse model
	N-azolylacrylate analogs (21)	Small molecule	HIV model
	CBS9106 (21)	Small molecule	Xenograft mouse model
	SINE series (110)	Small molecule	Phase I–II clinical trials

[a]*As published until December 2015.*

targeting cellular machinery that is active in both normal and cancer cells. However, studies have shown that cancer cells are more sensitive to nuclear transport inhibition than non-cancer cells which remain viable (*3*, (5,99)). Specific knock-down using siRNA for nuclear import proteins, KPNB1 and KPNA2, and export proteins, XPO1 and CSE1L, in cancer cells frequently results in reduced proliferation and increased apoptosis (50,57,78,96,98,100–102). This suggests that cancer cells may have an enhanced dependence on nuclear transporters for their increased proliferative and metabolic demands (referred to as tumour cell "addiction").

Targeting Nuclear Export via XPO1

Among the Karyopherin family members, the nuclear exporter, XPO1, has so far been the most successful chemotherapeutic target already being tested in clinical trials (Table 2). Several natural products were first described as inhibitors of XPO1 with Leptomycin B being the most potent. Unfortunately, Leptomycin B showed severe cytotoxic effects in phase I clinical trials and was no longer pursued for use in patients. Following that several Leptomycin B derivatives such as Anguinomycin and Kos-2464 have been synthesized in an attempt to reduce the cytotoxity while retaining the ability to inhibit nuclear export via XPO1, but have as yet not entered clinical trials. A recent review by Ishizawa et al. outlines the history of XPO1 as a chemotherapeutic target in more detail (21). In 2012 the SINE (selective inhibitors of nuclear export) series of drugs were discovered through an *in silico* molecular modelling strategy and synthesized by Karyopharm Therapeutics (Karyopharm Therapeutics, Boston, MA) (110). These compounds are water-soluble and irreversibly modify a cysteine (Cys528) in the NES-binding groove of XPO1, thereby inhibiting the function of the protein. KPT-185 is the most potent of the series and is most commonly studied *in vitro*; however, KPT-330/Selinexor, while nearly as potent, has more acceptable pharmacokinetics and has shown promise in phase I and II clinical trials in both haematological and solid tumours (111).

More recently, combination treatment strategies using XPO1 inhibitors and currently available chemotherapeutic agents have proven to be effective in enhancing the treatment of cancer. Nuclear export inhibitors alone, while effective in causing cell death *in vitro*, in animal models these inhibitors while inhibiting tumour growth were less often likely to eliminate existing tumours. The combination therapy is thought to use the XPO1 inhibitor to sensitise cancer cells to chemotherapeutic agents. A review by Turner et al. extensively discusses

a range of studies providing evidence for the efficacy of XPO1 inhbitors in combination with alkylating agents, anthracyclines, BRAF inhibitors, platinum drugs, protease inhibitors and tyrosine-kinase inhibitors against both haematological and solid tumours (112). SINE compounds, KPT-185, KPT-249 and KPT-330, were able to sensitize myeloma cells to doxorubicin, bortezomib and carfilzomib and phase I/II clinical are currently underway (112,113). A combination therapy using KPT-330 and gemcitabine has also been found to synergistically enhance cell death *in vitro* and *in vivo* in pancreatic cancer (114).

Targeting Nuclear Import via KPNB1 and KPNA

The field of nuclear import inhibitors, while not as advanced as that of nuclear export inhibitors at present, is growing (Table 2). Most commonly, KPNB1 and the KPNA isoforms work together to transport cargoes into the nucleus, although KPNB1 can also transport cargo independently. Therefore, targeting KPNB1 may have a broader spectrum of import inhibition while targeting a single KPNA isoform would considerably increase specificity. Pioneering work in the search for Karyopherin alpha/beta 1 inhibitors was started by Lin et al. in 1995. They, among others, found that a cell-permeable peptide containing the NLS of the NFκB p50 subunit was able to inhibit the nuclear import of transcription factors containing a NLS, such as NFκB, NFAT, AP-1 and STAT1 (115,116). The peptide became better known as cSN50.1 and more recently has been found to target both SREBP/KPNB1- and KPNA/NLS-cargo-mediated import through two separate mechanisms. The inhibition of SREBP/KPNB1-mediated import is thought to be through the binding of the peptide's SSHR motif with KPNB1. The peptide is also able to bind all KPNA isoforms, except KPNA6, with the highest affinity for KPNA1 and in that way plays a role in the import inhibition of transcription factors containing a NLS (103). Kosugi et al. also used peptide inhibitor design to target the KPNA/B1 import pathway which yielded two high-affinity peptides namely; Bimax1 and Bimax2. They were found to specifically target the KPNA-mediated import pathway and had no effect of KPNB1-only mediated import although they showed no specificity for Karyopherin alpha isoforms in mammalian cells (105). Unfortunately, the chemotherapeutic ability of these import inhibitors remains unknown. A peptide, M9M, has also been designed to inhibit nuclear import but rather by targeting TNPO1 which recognises a PY-NLS (108). M9M was used to elucidate the role of TNPO1 in mitosis but its role in cancer has not been explored (75). An antiparasitic antibiotic, Ivermectin, already on the market, has also been found to bind non-specifically to KPNB1/KPNA preventing binding to the cargo proteins (104). Although Ivermectin has been shown to have anticancer effects this seems to be through a mechanism of action unrelated to nuclear import inhibition (117).

Karyostatin 1A was the first small-molecule inhibitor of KPNB1-mediated nuclear import to be described. Its mechanism of action is thought to be through blocking the binding of RanGTP to KPNB1 disrupting the KPNA/B1 import pathway (106). The potential of this compound including its anticancer effect has not been explored to date. Another small-molecule inhibitor of nuclear import, Importazole, developed by Soderholm et al., was found to interfere with the interaction between RanGTP and KPNB1 and specifically disrupts KPNB1-mediated import without affecting Transportin or XPO1-mediated transport (107). Importazole successfully inhibited NFκB p65 nuclear localisation in myeloma cells and induced apoptosis without affecting normal plasma cells, showing promise as an anticancer compound (118). Although no studies have been published using nuclear import inhibitors in combination with currently available chemotherapeutic agents it may be an area of interest following the potential success of combination therapies with nuclear export inhibitors.

Although other members of the Karyopherin family, such as CSE1L, show promise as cancer therapeutic targets, no inhibitors are available for any of the other transport proteins as yet. The targeting of other nuclear transport proteins that have a more limited selection of cargoes may have value as anticancer strategies. For this to occur, their association with cancer requires further investigation.

Conclusions and Perspectives

Nuclear transport proteins play an essential role in cellular functioning. Hence, the increased reliance, of transformed and cancer cells, on the nuclear transport proteins for their increased metabolic demands warrants their usefulness as chemotherapeutic targets. More recently, the potential of nuclear transporters as cancer biomarkers has been investigated. For example, the secretion of certain Karyopherin proteins into the urine or serum of cancer patients shows diagnostic potential, while the differential expression of Karyopherin proteins in cancer tissue in comparison to normal tissue suggests that these proteins have potential as prognostic markers as well. Further research is required to elucidate the clinical potential of nuclear transport proteins as diagnostic, prognostic and therapeutic targets.

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